Published April 15, 1993

Thyroid Hormone Receptor/c-*erb*A: Control of Commitment and Differentiation in the Neuronal/Chromaffin Progenitor Line PC12

Alberto Muñoz,* Christopher Wrighton,‡ Barbara Seliger,§ Juan Bernal,* and Hartmut Beug‡

*Instituto de Investigaciones Biomédicas, Consejo Superior de Investigaciones Cientificas (CSIC) 28029 Madrid, Spain; ‡Institute of Molecular Pathology, Dr. Bohrgasse 7, A 1030 Vienna, Austria; and §III Medizinische Klinik, Abt. Hämatologie/Onkologie D 6500 Mainz, Germany

Abstract. The c-*erb*A proto-oncogenes encode nuclear receptors for thyroid hormone (T_3), a hormone intimately involved in mammalian brain maturation. To study thyroid hormone receptor (TR) action on neuronal cells in vitro, we expressed the chicken c-*erb*A/TRα-1 as well as its oncogenic variant v-*erb*A in the adrenal medulla progenitor cell line PC12. In the absence of T_3, exogenous TRα-1 inhibits NGF-induced neuronal differentiation and represses neuron-specific gene expression. In contrast, TRα-1 allows normal differentiation and neuronal gene expression to occur in the presence of T_3. Finally, TRα-1-expressing cells become NGF-responsive for proliferation when T_3 is absent, but NGF-dependent for survival in presence of T_3. A similar differentiation induction by NGF plus T_3 was observed in a central nervous system-derived neuronal cell line (E 18) expressing exogenous TRα-1. Together with the finding that TRα-1 constitutively blocked dexamethasone-induced differentiation of PC12 cells into the chromaffin pathway, these results suggest that TRα-1 plays an important role in regulating commitment and maturation of neuronal progenitors.

In contrast, the v-*erb*A oncogene, a mutated, oncogenic version of TRα-1, partially but constitutively inhibited NGF-induced neuronal differentiation of PC12 cells and potentiated dexamethasone-induced chromaffin differentiation, giving rise to an aberrant "interlineage" cell phenotype.

THYROID hormone (3,5,3'-triiodo-L-thyronine, T_3)[1] is an essential regulator of physiological and developmental processes in many cells and tissues. In particular, T_3 plays a crucial role in late embryonic and neonatal development of the brain (for reviews see Hamburg, 1969; Morreale de Escobar et al., 1983; Legrand, 1984; Dussault and Ruel, 1987). Deprivation of thyroid hormone during rat brain development causes diverse histological and cellular abnormalities such as changes in cell populations, decreases in axon densities, dendrite formation and myelin deposition, and alterations of cell migration, leading to severe impairment of brain maturation and function. These alterations are reversible by T_3 replacement until a certain age and might reflect aberrant or even absent differentiation of both neurons and glial cells. In humans, permanent mental deficiency due to insufficient T_3 levels can only be avoided if replacement therapy is started early after birth. In spite of the crucial importance of thyroid hormone for proper development of the central nervous system (CNS), very little is known about the molecular basis of thyroid hormone receptor action in neuronal cells, most likely due to the complexity of the CNS and the asynchronic patterns of development among their different parts and cell groups. Only recently, a few genes were found to be regulated at the pretranslational level by thyroid hormone in vivo (Muñoz et al., 1991).

After identification of the c-*erb*A proto-oncogene as the thyroid hormone receptor (TR) α-1 (Sap et al., 1986; Weinberger et al., 1986) and discovery of two *erb*A genes in higher organisms (α and β) encoding a whole family of different receptor proteins, several groups detected *erb*A expression in the brain. CNS neurons express the thyroid hormone receptor, and an increase in T_3-binding sites correlates with the period of neuroblast proliferation in human fetuses (Bernal and Pekonen, 1984). TRα RNA has been detected very early (day 4) in the embryonal chicken brain, increasing progressively thereafter during the embryonal period (Forrest et al., 1990, 1991). TRβ appears later in embryogenesis, becoming predominant in certain areas of both the CNS and the eye (Forrest et al., 1991). During rat brain maturation, both α and β receptors are expressed in a distinct fashion characterized by tight temporal and spatial regulation (Bradley et al., 1989; Strait et al., 1991; Mellström et al., 1991). In situ hybridization studies indicate that TR mRNAs are present mainly in neurons from those brain areas known to be targets of thyroid hormone, such as the cerebral cortex, hippocampus, cerebellum, and basal ganglia. In particular, cholinergic cells of the basal forebrain are

1. *Abbreviations used in this paper*: CNS, central nervous system; DEX, dexamethasone; RA, retionic acid; RAR, retionic acid receptor; TH, tyrosine hydroxylase; TR, thyroid receptor; T_3 triiodothyronine.

Published April 15, 1993

sensitive to thyroid hormones (Gould and Butcher, 1989). These cells also express maximum levels of the high affinity ($gp140^{trk}$) NGF receptor (Holtzman et al., 1992) and thyroid hormone and NGF were found to cooperate in the development of specific cholinergic markers in basal ganglia (Patel et al., 1988) and other CNS areas (Clos and Legrand, 1990). These findings strongly suggest a key role of thyroid hormone receptors in neural tissue formation and maturation, probably involving cooperation with neurotrophins.

We have addressed this question by studying the effects of T_3 on neuronal cells differentiating in culture. Unfortunately, appropriate neuronal cell lines from the CNS capable of in vitro differentiation in response to neurotrophic factors are missing and primary cells obtained from brain do not grow in culture for extended time periods, hampering long-term studies. We therefore employed the PC12 cell line that has been extensively used as a model system for neuronal differentiation since its establishment from a rat pheochromocytoma (Greene and Tischler, 1976). PC12 cells show characteristics of common precursor cells for sympathetic adrenergic neurons and neurosecretory epithelioid chromaffin cells. NGF induces PC12 cells to differentiate along the neuronal pathway, whereas glucocorticoid hormones promote their differentiation into chromaffin cells (Greene and Tischler, 1982; Levi et al., 1988). Thus, PC12 differentiation in vitro at least partially mirrors differentiation of respective neural crest-derived progenitor cells in the rat adrenal medulla in vivo (Doupe et al., 1985*a*,*b*; Anderson and Axel, 1986).

Because of their origin from a tumor, it was not surprising that PC12 cells behave aberrantly in several ways when compared to normal precursor cells in vivo. They continuously grow in the absence of neurotrophic factors, show an altered response to various exogenous stimuli and differentiate only partially into either neuronal or chromaffin cells. Also, they show no known response to thyroid hormone, a result also obtained in our own pilot experiments. This could be due to expression of low levels of c-*erb*A/TRα-1 together with high levels of c-*erb*A/TRα-2 RNA, which encodes a variant form of the receptor generated by differential splicing. TRα-2 is unable to bind hormone but efficiently blocks transcriptional activation by the active c-*erb*A/TRα-1 form (Koenig et al., 1989; Lazar et al., 1989; Rentoumis et al., 1990).

For these reasons, we decided to stably express an exogenous TRα-1 gene in PC12 cells using suitable retroviral vectors (Muñoz et al., 1990). The rationale behind this study was to restore TRα-1 expression in a neuronal progenitor cell line that express only low levels of TRα-1, most likely as a consequence of the immortalization process, and then analyze neuronal differentiation in response to thyroid hormone and neurotrophic factors. We show in this paper that exogenous TRα-1 expressed at physiological levels regulates NGF-dependent differentiation and gene expression of PC12 cells in a T_3 dependent fashion and constitutively blocks their entry into the chromaffin differentiation pathway. In addition, ligand-activated TRα-1 renders PC12 cells dependent on NGF as a neurotrophic factor. In contrast to TRα-1, v-*erb*A, a mutated, oncogenic version of TRα-1 that seems to act as a constitutive repressor of genes regulated by c-*erb*A/TRα-1 (Damm et al., 1989; Sap et al., 1989; Disela et al., 1991), constitutively inhibited both neuronal and chromaffin differentiation of PC12 cells.

Materials and Methods

Cells and Cell Cultures

A clonal subline of rat PC12 cells (referred to as 6c) was used throughout. This clone responded particularly well to differentiation induction by NGF in terms of neurite outgrowth on standard plastic dishes. (Wrighton, C. W., and M. Busslinger, unpublished observations). PC12 6c cells were grown in DME supplemented with 10% horse serum, 5% FCS, and 1 mM glutamine (standard growth medium) (all from GIBCO, U.K.). Establishment and properties of the E 18 cell line derived by spontaneous immortalization from 17-d embryonic rat brain will be described elsewhere (Seliger, B., unpublished observations). Cells were grown in Ham's F12 medium supplemented with 10% FCS.

Retrovirus Vectors and Generation of erbA-overexpressing Cells

The recombinant retroviruses encoding the chicken c-*erb*A/TRα-1 and v-*erb*A genes used to infect PC12 6c cells have been already described (Muñoz et al., 1990). Both are derived from the Moloney murine leukemia virus and encode the neomycin-resistant gene providing resistance to the antibiotic G418. For infection, 5×10^5 cells were plated in 60-mm dishes and incubated overnight with fibroblast-grown virus plus 8 μg/ml polybrene (Sigma Chem. Co., St. Louis, MO). Medium was then changed and selection with 800 μg/ml G418 (GIBCO BRL, Gaithersburg, MD) started 48 h after infection (Muñoz et al., 1990). Six clones of resistant TRα-1-infected cells could be expanded separately (cA1–cA6). cA4, the clone exhibiting the highest specific T_3-specific binding was used in all experiments. In contrast, no individual v-*erb*A-infected clones could be expanded. Therefore, a pool of G418 resistant, v-*erb*A-expressing clones was used.

Infection of E 18 cells with TRα-1 was done exactly as described for PC12 6c. Ten TRα-1-expressing clones were obtained, one of which showing reproducible, high TRα-1 expression as determined by T_3 binding (E 18 cA9) was used in the experiments.

Induction of Neural and Chromaffin Differentiation

For induction of optimal neuronal differentiation, well suspended PC12 6c cells were seeded at 10^4 cells per cm^2 in standard growth medium. Murine 7 S NGF (Sigma Chem. Co.) was added at a concentration of 50 ng/ml every 2 d. Triiodothyronine (Sigma Chem. Co.) was added daily at a final concentration of 150 nM. Medium was changed partially or totally every 48 h. Chromaffin differentiation was induced by treatment with dexamethasone (5 μg/ml; Serva, Heidelberg) every 2 d or when the medium was changed.

Embryonic CNS neuroblasts (E 18 cA9) were induced to differentiate by seeding them in serum-free Dulbecco's plus Ham's F12 (1:1) medium supplemented with the additions of N_2 differentiation medium (Bottenstein and Sato, 1979). NGF (50 ng/ml) was added every 2 d while T_3 (150 nM) was added daily.

[^{3}H]Thymidine Incorporation Assay

Cells were seeded into 35-mm dishes in 2 ml standard growth medium at a density of 8×10^4 cells per dish and treated with NGF and/or T_3 as described above. Cell monolayers were labeled for 3.5 h with 1 μCi [6-^{3}H] thymidine (25 Ci/mmol: Amersham Intl., Buckinghamshire, U.K.), washed twice with PBS, precipitated with 10% trichloroacetic acid for 10 min, air-dried, and dissolved in 400 μl of 0.1% NaOH and 1% SDS. Radioactivity in 200 μl was estimated in a β counter.

Mitomycin-C Treatment

Cells were treated for 2 h with mitomycin-C (Sigma Chem. Co.) at a final concentration of 20 μg/ml. In pilot experiments, this concentration inhibited thymidine incorporation to <1% of respective controls without producing toxic effects. Monolayers were then washed three times with PBS, incubated for 30 min with fresh medium, washed again, and then trypsinized and reseeded for differentiation induction. Except for different mitomycin-C concentrations, the same procedure was previously used to demonstrate that differentiation occurs in absence of cell division in myoblasts (Falcone et al., 1984), macrophages (Beug et al., 1987), and erythroid cells (Beug et al., 1992).

Published April 15, 1993

Immunoprecipitation

Detection of *erb*A protein ($p46^{c\text{-}erbA}$ and $p75^{gag\text{-}v\text{-}erbA}$) by [^{35}S]methionine labeling and immunoprecipitation analysis was done as described earlier (Goldberg et al., 1988; Glineur et al., 1990) using an anti-v-*erb*A antibody generously donated by Dr. J. Ghysdael.

Hormone Binding Assay

T_3-specific binding assays in vivo were performed as previously described (Sap et al., 1986; Muñoz et al., 1990). Specific binding is defined as the ratio of iodinated T_3 bound in absence and presence of a 1,000-fold molar excess unlabeled T_3.

Detection of Neuronal and Chromaffin Differentiation Antigens by Immunofluorescence

Fixed and permeabilized cells were analyzed by indirect immunofluorescence using monoclonal antibodies to synaptophysin, tyrosine hydroxylase, glial fibrillary acidic protein (all from Boehringer Mannheim Corp., Germany), and N-CAM (Sigma Chem. Co.) according to standard procedures. Rabbit antiserum to mouse E-cadherin (uvomorulin; generously provided by Dr. R. Kemler) and mouse 46-kd cytokeratin (provided by Dr. E. Reichmann) were used similarly. Basically, cells were fixed with 3.7% paraformaldehyde plus 0.01% glutaraldehyde at 4°C for 15 min, and then permeabilized with 0.1% NP-40 in medium for an additional 15 min at room temperature. Antibodies were used at the dilutions recommended by the manufacturers in a final volume of 50 μl. After a 1-h incubation at room temperature, the plates were washed with PBS and stained with second antibody (FITC-conjugated anti-mouse or anti-rabbit IgG, Amersham, diluted 1:50) for 1.5 h. After staining, cells were washed three times with PBS, mounted in Mowiol (HOECHST), and viewed using a Zeiss Axiophot fluorescence microscope with epiillumination.

RNA Preparation and Northern Analysis

Total RNA was prepared using the guanidinium isothiocyanate-phenol-chloroform procedure (Chomczynski and Sacchi, 1987). Purification of poly(A)$^+$ RNA was done as described (Vennström and Bishop, 1982). Northern blots were performed on Gene Screen membranes (New England Nuclear, Boston, MA) according to standard protocols (Maniatis et al., 1982). All probes were labeled by the random priming method (Feinberg and Vogelstein, 1983) using commercial kits (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). Source of probes: rat c-*erb*A/TRα-1 from Dr. R. M. Evans, rat TR-β from Dr. H. C. Towle, mouse c-*jun*, *junB*, and *junD* and integrin-β1 from Dr. R. Bravo, rat *fra*-1 from Dr. T. Curran, rat NGFI-A and NGFI-B from Dr. J. Milbrandt, v-*fos* from Dr. E. Wagner, rat transin from Dr. R. Breathnach, mouse collagen α1 (I) from Dr. Z. Q. Wang, rat SCG10 from Dr. D. J. Anderson, rat GAP-43 from Dr. M. C. Fishman, rat tyrosine hydroxylase from Dr. E. Ziff, rat chromogranin B from Drs. S. Forss-Petter/J.G. Sutcliffe, mouse N-CAM from Dr. C. Goridis, mouse cytokeratin (endoB/K18) from Dr. M. Busslinger, human trk from Dr. M. Barbacid, mouse trk from Dr. L. Parada, rat LNGFR from Dr. E. M. Shooter, human RARα and mouse RARβ from Dr. H. Stunnenberg, and rat NF68 from Dr. F. Grosveld. Rat MASH-1 and MASH-2 genes were cloned from poly (A)$^+$ RNA prepared from dense cultures of PC12 6c cells by cDNA synthesis and PCR amplification using primers specific for the extremes of the published sequences (Johnson et al., 1990) obtained from the EMBL data bank. The oligonucleotides used were 5'AATATAATG-AAGCTTGCGCGTTTCTTCCCTTTTAAC 3' and 5'TATGATCAAGAA-TTCCATCCTGCTTCCAAAGTCCAT 3' for MASH-1 and 5'AATATAATG-AAGCTTGTCGAGGACGCATTAAGC 3' and 5'TATGATCAAGAATTC-CAAGTCCTGGTTTCGGCAA 3' for MASH 2. The amplified products were cloned into the HindIII and EcoRI sites of the pRK7 plasmid. Fragments covering the nonhomologous 3' ends were prepared as specific probes for both genes. A 308-bp PvuII fragment was prepared for MASH-1 and a 397-bp PstI-ScaI fragment for MASH-2. Hybridizations were carried out overnight at 65°C in 7% SDS, 500 mM sodium phosphate buffer, pH 7.2, and 1 mM EDTA according to Church and Gilbert (1984). Filters were washed twice for 30 min each in 1% SDS and 40 mM sodium phosphate buffer, pH 7.2, at 65°C. Before rehybridizing the nylon membranes with probes for other genes, the radioactive probe was stripped off the membrane by placing it in a 75°C water bath for 5 min.

Results

Stable Expression of Exogenous erbA Genes in PC12 Cells

To analyze the possible role of T_3 and its receptor on PC12 differentiation in vitro, we sought to stably express the avian c-*erb*A/TRα-1 protein as well as its oncogenic variant, v-*erb*A, in these cells. Cultures of a clonal PC12 subline (6c, see Materials and Methods) were infected with retroviruses expressing c-*erb*A/TRα-1 or v-*erb*A together with the neo^r gene as a selectable marker. Several neoresistant clones of c-*erb*A/TRα-1 infected cells were obtained. In contrast, we only succeeded to expand a pool of v-*erb*A-infected cell clones due to their poor clonal proliferation ability.

The expression of the respective TRα-1 and v-*erb*A proteins was monitored by immunoprecipitation using anti-*erb*A antisera. c-*erb*A/TRα-1 protein was just detectable, whereas v-*erb*A was expressed at high levels, comparable to those found in avian erythroleukemic cells (Fig. 1 *A*). Synthesis of active c-*erb*A/TRα-1 protein was confirmed by a hormone binding assay after incubation with ^{125}I-labeled T_3. Specific binding was one order of magnitude higher in c-*erb*A/TRα-1-expressing cells than in uninfected or v-*erb*A-expressing cells (Fig. 1 *B*), and of the same order of magnitude as that of rat GH1 pituitary cells containing a few thousand active receptor molecules per cell (Samuels et al., 1976, 1977).

Endogenous Hormone Receptor Expression in Normal and erbA-expressing PC12 Cells

Since several cell lines of neural origin express the thyroid hormone receptor variant TRα-2 in high excess over TRα-1 (Muñoz, A., J. Bernal, and A. Rodriquez-Peña, unpublished observations), we tested if this was also true for PC12. As shown by Northern blot analyses, normal PC12 cells expressed much higher levels of the TRα-2 form than of the TRα-1 and TRβ receptors (Fig. 1 *C*). In contrast, the retrovirus transduced exogenous c-*erb*A/TRα-1 and v-*erb*A genes were expressed at levels comparable to those of the endogenous TRα-2 receptor. Thus, the nonresponsiveness of PC12 cells to T_3 may in fact be due to functional suppression of the low TRα-1 levels by an excess of TRα-2.

To rule out that the c-*erb*A/TRα-1- and v-*erb*A-expressing PC12 sublines were altered in their expression of other important receptors, the same blots were hybridized with cDNA probes encoding the low ($gp80^{LNGFR}$) and high ($gp140^{trk}$) affinity NGF receptors as well as the retinoic acid receptors (RARs) α and β. All these receptors were expressed in the parental cells (distinct signal from 10 μg total RNA after 5-d exposure) and no changes in expression levels were detected in the sublines expressing exogenous *erb*A proteins (data not shown).

Ligand-activated TRα-1 Renders PC12 Cells NGF-dependent for Proliferation and Survival

Neurotrophic factors such as NGF are considered to be necessary for survival of differentiating neuronal cells, including primary adrenal medulla sympathetic neurons in culture. (Doupe et al., 1985*b*; Anderson and Axel, 1986). In contrast, PC12 cells grow in the absence of NGF, and revert to an undifferentiated state if the factor is withdrawn af-

Published April 15, 1993

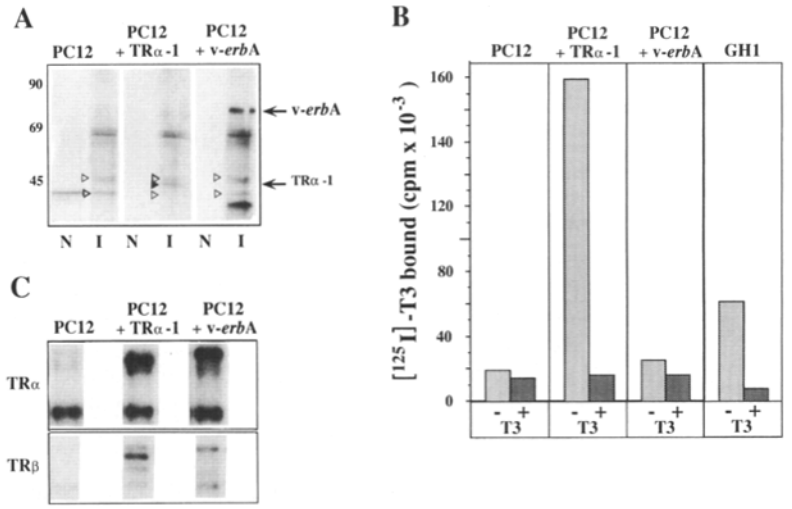

Figure 1. ErbA expression in PC12 cells. (*A*) Uninfected, TRα-1/c-*erb*A- and v-*erb*A-infected PC12 cells were labeled with [^{35}S]methionine and lysates from equal numbers of cells were immunoprecipitated with an antibody against v-*erb*A. The immunoprecipitates were analyzed on 10% SDS-polyacrylamide gels and fluorographed. Films were exposed for 6 d. Bands corresponding to the chicken p46$^{c\text{-}erbA}$ and the viral p75$^{gag\text{-}v\text{-}erbA}$ are indicated by arrows. The faint p46$^{c\text{-}erbA}$ band is also indicated by a solid triangle to distinguish it from two bands precipitated nonspecifically (*open triangles*). (*N*) preimmune serum; (*I*) immune serum. (*B*) Uninfected and infected PC12 cells were analyzed for T3-specific binding as described (Sap et al., 1986; Muñoz et al., 1990), in presence (+T_3) or absence (−T_3) of a 1,000-fold molar excess (0.3 μM) of cold T_3 as competitor. Rat pituitary GH1 cells expressing high levels of endogenous T_3 receptors were used as a control. (*C*) Analysis of TR RNA expression by Northern blots in uninfected, TRα-1- and v-*erb*A-infected PC12 cells. 10 μg of total RNA were analyzed per lane. Exposure times: rat c-*erb*A/TR-α, 6 d; rat c-*erb*A-TR/β, 14 d. Mature endogenous TRα-1 RNA (*lower band*, 5 kb) and its precursor (*upper band*, 6.5 kb) are indicated with asterisks (*). The band corresponding to the endogenous TRα-2 RNA (2.6 kb) is marked with an (x). It coincides with that of the viral subgenomic RNA encoded by the internal promoter. This RNA, as well as the large unspliced viral genomic RNAs (indicated by *arrows*) crosshybridize with both rat c-*erb*A probes, α and β. Hybridization with the β probe gives two very weak signals corresponding to the precursor (6.5 kb, *arrowhead*) and mature (4 kb) RNAs.

ter induction of differentiation. To determine whether TRα-1 expression would affect PC12 cells responsiveness to NGF, we first studied the effects of T_3, NGF, or their combination on the capacity of uninfected and *erb*A expressing cells to proliferate and to synthesize DNA.

As shown in Fig. 2, treatment with NGF alone or NGF plus T_3 did not grossly affect proliferation of uninfected cells (panel *A*, left). In cells expressing exogenous TRα-1 however, T_3 and NGF had clear but opposite effects on survival and proliferation. NGF alone led to a drastic increase

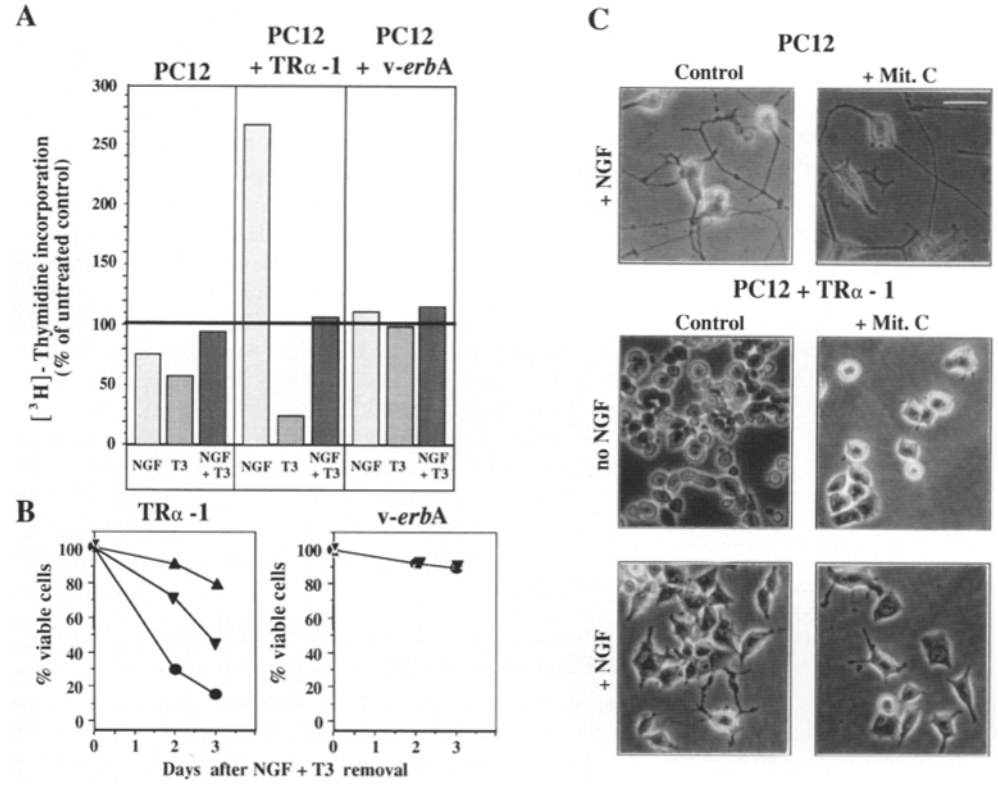

Figure 2. Effects of T_3 and NGF on the proliferation and survival of uninfected, TRα-1- and v-*erb*A-PC12 cells. (*A*) Proliferation capacity of the three cell types was analyzed after 5 d of treatment with NGF, T_3, or both hormones (NGF + T_3) by measuring the incorporation of tritiated thymidine into trichloroacetic acid–precipitable material (see Materials and Methods). Horizontal bar indicates the incorporation level (100%) of the corresponding untreated control cells. (*B*) TRα-1- and v-*erb*A-PC12 cells were treated for 7 d with NGF plus T_3, and then incubated with T_3 alone (*circles*), NGF alone (*triangles*), or no hormone (*inverted triangles*). Surviving cells were counted 2 and 3 d later after extensive washing of the monolayers. Hundred percent values correspond to cell numbers obtained with each cell type after the initial 7-d NGF plus T_3 treatment. (*C*) TRα-1-PC12 cells were incubated with mitomycin C (*Mit. C*, see Materials and Methods) or left untreated (*Control*). They were then incubated with NGF for 6 d (+ *NGF*) or with NGF-free medium (*no NGF*). Phase micrographs of these cells (*middle and lower panels*) as well as of respective uninfected PC12 cells induced to differentiate with NGF are shown (*upper panels*). Bar, 50 μm.

Published April 15, 1993

in the level of DNA synthesis (Fig. 2, panel *A*, middle) which was reflected by a 30% increase in cell number after 5 d (not shown). T_3 had an opposite effect: DNA synthesis ceased, and cells no longer divided and degenerated progressively. None of these effects were observed upon combined treatment with NGF and T_3.

These observations suggested that ligand-activated TRα-1 indeed rendered PC12 cells dependent for survival and proliferation on neurotrophic factors like NGF. To confirm this, cells were pretreated for one week with NGF + T_3, and then cultured in medium containing no additions of either T_3 or NGF separately. When switched to T_3 containing medium, more than 80% of TRα-1 cells degenerated within 3 d (Fig. 2 *B*), while they survived as differentiated cells in control cultures containing NGF + T_3 (see below). A significant proportion of TRα-1 cells died even in plain medium and only NGF was able to prevent cell death. V-*erb*A-expressing cells were completely refractory to any of these treatments (Fig. 2 *B*), while normal, uninfected cells showed weak, intermediate responses (data not shown). Whether or not the cells died by programmed cell death (apoptosis) after removal of NGF as shown to take place during normal CNS development in vivo (for review see Oppenheim, 1991) is still under investigation.

C-erbA/TRα-1 and v-erbA Affect NGF-induced Neuronal Differentiation in PC12 cells

To determine whether TRα-1 or v-*erb*A expression affects the ability of PC12 cells to differentiate into neurons in response to NGF, uninfected cells, and cells expressing c-*erb*A/TRα-1 or v-*erb*A were treated with NGF, T_3, or both for 5–7 d. The extent of neuronal differentiation was estimated by monitoring neurite outgrowth and expression of two neuronal markers, synaptophysin and N-CAM (Prentice et al., 1987; Mann et al., 1989), on neurites and cell bodies by immunofluorescence. As controls, cells were stained with antibodies to tyrosine hydroxylase (TH), an enzyme which is downregulated by NGF but induced by dexamethasone during chromaffin differentiation (Lewis et al., 1983; Leonard et al., 1987; Stein et al., 1988*b*), and to glial fibrillary acidic protein, an astrocyte marker.

As expected, NGF treatment of uninfected cells induced cell flattening followed by appearance and progressive elongation of neurites and rounding of cell bodies (Fig. 3). The neurites expressed high levels of synaptophysin and lower levels of N-CAM, while TH expression decreased (from 55 to 16% fluorescence positive cells; Fig. 3). As mentioned above, T_3 alone had no major effects on normal PC12 cells and did not detectably alter NGF-induced differentiation, when applied together with NGF (data not shown).

In contrast, expression of exogenous TRα-1 strongly arrested NGF-induced differentiation. When treated for 6 d with NGF alone, the cells developed only short processes that failed to elongate even after prolonged NGF application (Fig. 4). TRα-1-cells also failed to upregulate synaptophysin and N-CAM expression in response to NGF (Fig. 4 and data not shown). TH content was low already in the uninduced stage (20% positive cells) and was further reduced after NGF (2.5% positive cells, not shown).

Interestingly, the differentiation block could be completely reversed by addition of T_3 together with NGF. After 6 d of exposure to both agents, a high proportion of TRα-1 cells produced long processes expressing synaptophysin and N-CAM at similar or even higher levels as uninfected cells in the presence of NGF alone (compare Figs. 3 and 4, data not shown). In the absence of NGF, T_3 induced progressive cell death which affected 30–80% of the population after 5 d depending

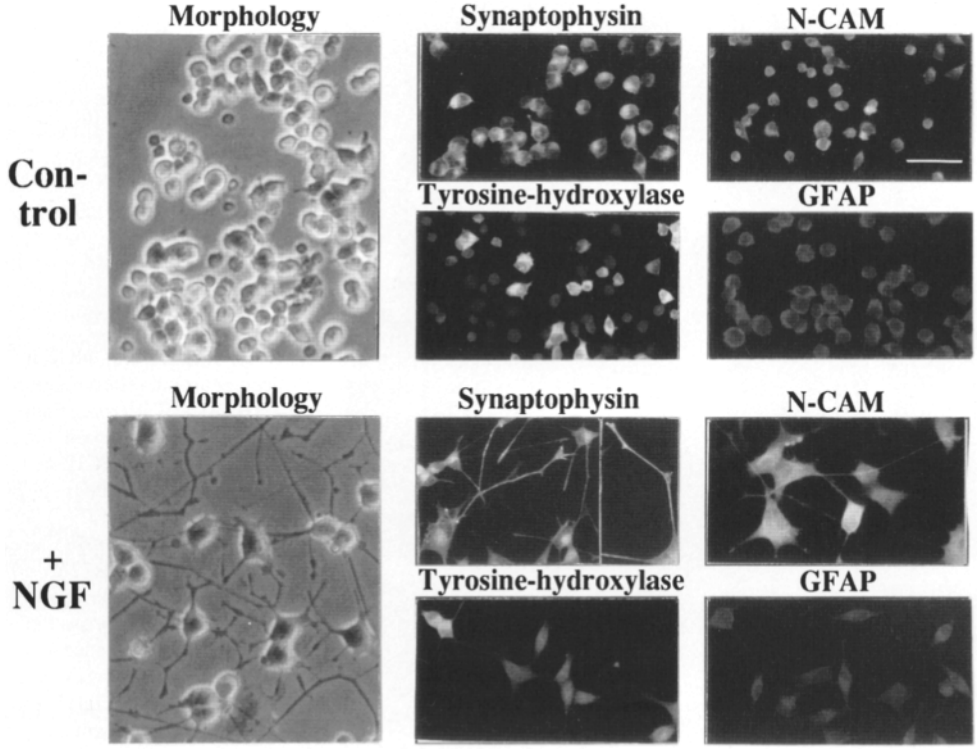

Figure 3. NGF-induced neuronal differentiation of clonal PC12 6c cells. Phase micrographs of uninfected PC12 6c cells treated with NGF for 7 d (+ *NGF*) see Materials and Methods) or left untreated (*Control*) are shown (*left panels*). Parallel cultures were stained with antibodies against synaptophysin, tyrosine hydroxylase, N-CAM, and glial fibrillary acidic protein (GFAP, negative control), and photographed (see Materials and Methods). Bar, 50 μm.

Published April 15, 1993

on the clone analyzed (Fig. 4, lower panels and data not shown).

Cells expressing v-*erb*A showed a distinctly different response. They were arrested in neuronal differentiation regardless of whether the cells were treated with NGF alone or in combination with T_3 (Fig. 4). In both cases, they formed only short processes that expressed no synaptophysin with NGF alone, while some expression was seen at the neurite tips with NGF + T_3 (Fig. 4). This latter result agrees with the assumed role of v-*erb*A as a constitutive repressor of TR-regulated genes (Sap et al., 1989; Damm et al., 1989). T_3 alone had no effect on these cells.

Since cells expressing TRα-1 were induced by NGF to grow at an increased rate we had to rule out that their differentiation arrest was a consequence of an increased proliferation and cell density. To rule out this possibility cell differentiation was studied after inhibition of cell proliferation with mitomycin-C. Uninfected PC12 cells differentiated normally in response to NGF in presence or absence of mitomycin-C (Fig. 2 *C*, upper panels). In contrast, both mitomycin-treated and control TRα-1 cells formed only short processes in response to NGF (Fig. 2 *C*). This suggests that the stimulation of proliferation and the differentiation arrest caused by TRα-1 in NGF-treated PC12 cells are unrelated phenomena.

Retinoic Acid Receptors Do Not Play a Major Role in TRα-1 Regulated Neuronal Differentiation of PC12 Cells

Since PC12 cells express retinoic acid receptors (RARα and RARβ) and cooperation of TRs and RARs is thought to be crucial for thyroid hormone action in several systems (Hudson et al., 1990; Schroeder et al., 1992*b*), we determined whether retinoic acid (RA) would affect T_3-dependent regulation of differentiation in normal and TRα-1-expressing PC12 cells. In either cell type, RA did not grossly affect neuronal differentiation induced by NGF or NGF + T_3. Also, RA did not alter the T_3-induced NGF dependence of TRα-1 cells. However, if used alone, RA seemed to induce cell rounding and formation of aggregates similar to those seen during the initial stages of dexamethasone-induced chro-

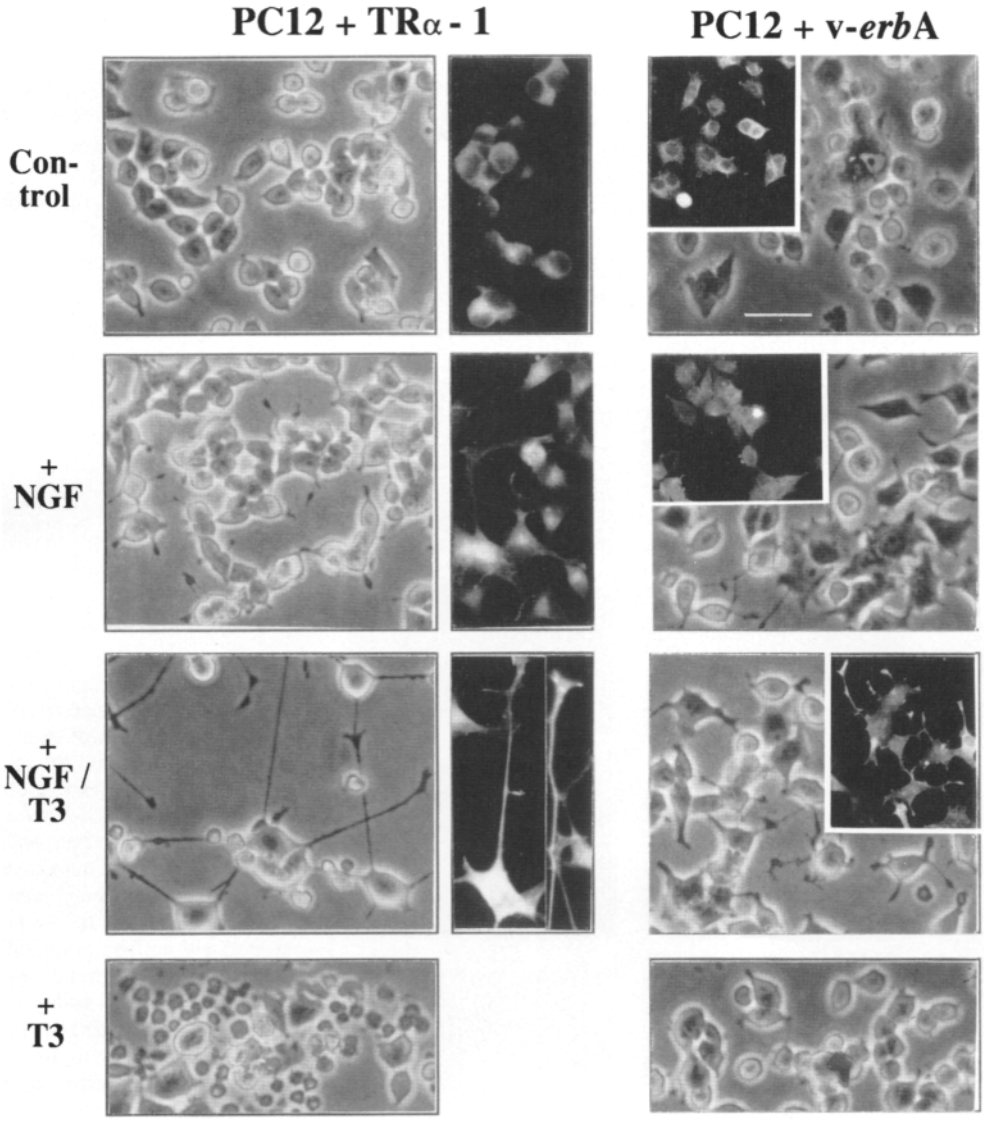

Figure 4. Arrest of NGF-induced neuronal differentiation by TRα–1 and v-*erb*A. Phase micrographs of TRα–1 and v-*erb*A-expressing PC12 cells treated with NGF, NGF plus T_3 (7 d) or T_3 alone (5 d), or left untreated (*Control*) are shown. Middle panels (*TRα–1*) and insets to right panels (*v-erbA*) show the corresponding immunofluorescence analysis using synaptophysin antibody (see Materials and Methods). Bar, 50 μm.

Published April 15, 1993

maffin differentiation (data not shown). Again, this response was not affected by the presence or absence of exogenous TRα-1, suggesting that the RARs do not play a major role in the regulation of neuronal differentiation by TRα-1.

TRα-1 Does Not Affect the Induction of Early Genes by NGF

Several dozen genes are known to be induced by NGF in PC12 cells (for a review see Halegoua et al., 1991). These genes can be distributed in two groups: early response genes whose induction begin a few minutes after NGF addition and peak one or a few hours later, and late response genes which are induced with slower kinetics and reach their maximum level of expression two, four, or more days after continuous NGF treatment.

First, we analyzed possible effects of TRα-1 on the expression of NGF-inducible early genes, such as c-*jun*, *jun*-B, *jun*-D, c-*fos*, *fra*-1, NGFI-A (Krox 24, *egr*-1, *zif*/268), and NGFI-B (*nur*/77, N10) which have been reported to be induced by NGF to a variable extent (Greenberg et al., 1985; Kruijer et al., 1985; Morgan and Curran, 1986; Milbrandt, 1987, 1988; Bartel et al., 1989; Szeberényi et al., 1990). The *fra*-1 gene was particularly interesting, since its constitutive expression apparently blocks induction of many of the other early genes (Ito et al., 1990). Uninfected and TRα-1-expressing cells were treated for 1 h with NGF, T_3 or both hormones, and subjected to Northern blot analysis, using poly(A)$^+$ RNA (Fig. 5).

NGF caused a similar transient upregulation of all these genes in uninfected and TRα-1-expressing cells with strong increments of mRNA levels after 1 h, and much less after 24 h. Furthermore, T_3 neither superinduced nor suppressed the induction of any of these genes by NGF in either cell type

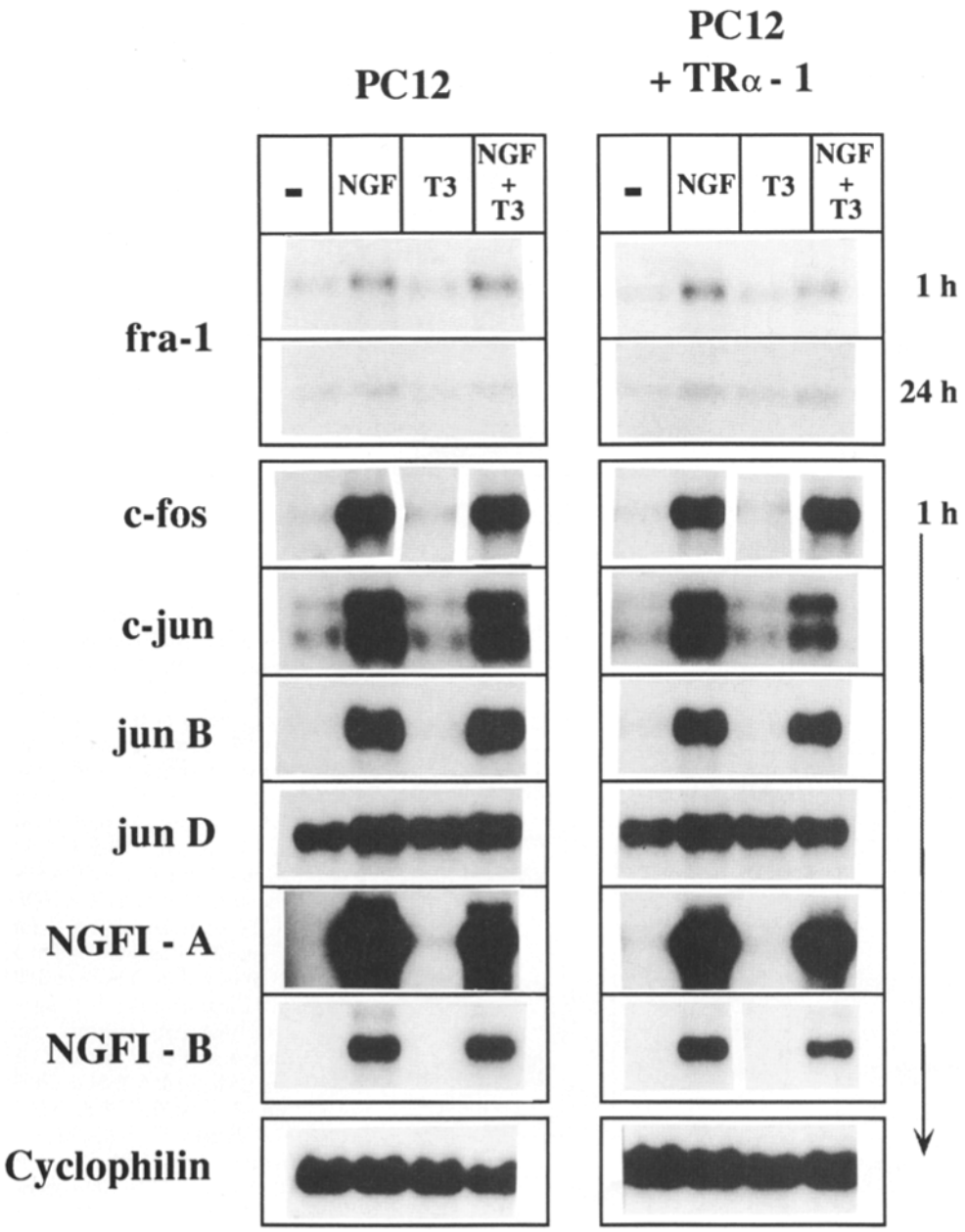

Figure 5. TRα-1 does not affect expression of NGF-induced early genes. Northern blot analyses for expression of NGF-induced early genes were performed with uninfected (*PC12*) and TRα-1-expressing PC12 cells (*PC12 + TRα-1*) treated for 1 (1 h) or 24 h (*24 h*) with NGF, T_3 or both (*NGF + T_3*), or left untreated (−). Expression of cyclophilin mRNA was used as control. 2.5 μg poly (A)$^+$ RNA were loaded per lane. Exposure times: *fra*-1, 3 d; *c-fos*, 3 d; *c-jun*, 1 d; *jun* B; 16 h; *jun* D, 16 h; *NGFI-A*, 4 h; *NGFI-B*, 16 h; and *Cyclophilin*, 16 h.

Published April 15, 1993

(Fig. 5 and data not shown). Similar patterns were obtained for ornithine decarboxylase (not shown). These results show that TRα-1 does not interfere at all with NGF-regulated early gene expression, indicating that NGF is able to induce all its normal intracellular signals in TRα-1-expressing PC12 cells.

TRα-1 Causes Hormone-dependent Repression of NGF-induced Late Genes

Having ruled out that TRα-1 affects NGF-dependent signal transduction, we checked the alternative hypothesis that TRα-1 might block the expression of neuronal-specific genes in absence of ligand, but allow or even enhance their NGF-regulated expression in presence of T_3. Such a result would be expected from a similar behavior of TRα-1 in transient transfection experiments (Damm et al., 1989; Sap et al., 1989) as well as in avian erythroblasts (Zenke et al., 1988, 1990). Consequently, cells were treated with NGF alone, NGF + T_3, or left untreated, and tested for late gene expression in Northern blots.

TRα-1 strongly affected expression of a large series of NGF-inducible late genes (Fig. 6). This group consisted of

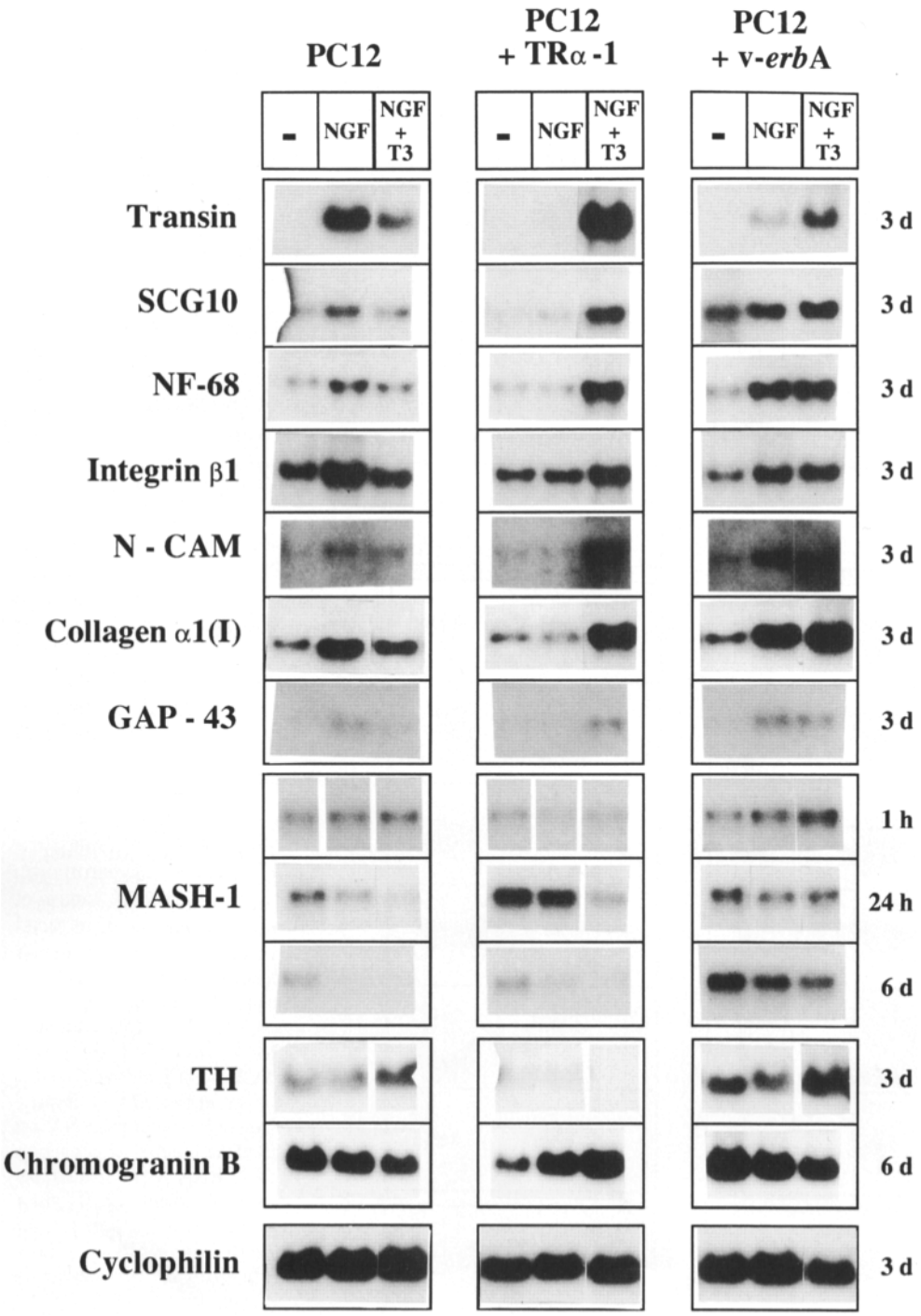

Figure 6. T_3-dependent regulation of NGF-induced late genes by TRα-1. Expression analysis of NGF-induced late genes (*upper panels*), MASH-1 (*middle panels*), and of two chromaffin marker genes (*lower panels*) by Northern blot hybridization was performed in uninfected, TRα-1- and v-*erb*A-expressing PC12 cells after treatment with NGF, NGF + T_3, or no hormone (−) for 1 and 24 h as well as for 3 (3 d) or 6 d (6 d) as described in the legend to Fig. 5. 10 μg of total RNA were loaded per lane. Exposure times: *MASH-1*, 1 and 24 h, 20 d; 6 d, 14 d; *Transin*, 3 d; *SCG10*, 3 d; *NF68*, 6 d; *Integrin* β_1, 6 d; *Collagen* α1(I), 4 d; *N-CAM*, 6 d; *GAP-43*, 6 d; *TH*, 16 h; *Chromogranin* B, 7 h; and *Cyclophilin*, 2 d.

Published April 15, 1993

four neuron-specific genes (SCG10, neurofilament 68, N-CAM, and GAP-43; Prentice et al., 1987; Stein et al., 1988*a*,*b*; Lindenbaum et al., 1988; Federoff et al., 1988), the protease transin probably involved in neurite outgrowth (Machida et al., 1989) and extracellular matrix proteins and their receptors (collagen-α1 [I], integrin-β1; Mann et al., 1989; Van Hoof et al., 1989; Costello et al., 1990). In absence of T_3, TRα-1 efficiently repressed upregulation of all these genes by NGF. Exposure of the cells to NGF plus T_3 stimulated expression of all genes to similar or even higher levels than observed in normal PC12 cells induced to differentiate by NGF alone or by NGF + T_3. In addition, several of these genes (e.g., SCG-10, integrin-β1) appeared to be repressed in PC12 cells expressing unliganded TRα-1 (Fig. 6). A similar pattern was found for two additional genes (peripherin and MAP-2) that were only weakly induced by NGF (data not shown). Thus, TRα-1 regulates neuronal gene expression in a fashion faithfully reflecting its effect on morphological differentiation.

TRα-1 Does Not Affect NGF-induced Repression of the Early Neuroblast Marker MASH-1

The MASH-1 and MASH-2 genes, rat homologues of the Drosophila *achaete-scute* genes probably involved in neuronal development, were recently isolated as candidates for neuronal "master" genes (Johnson et al., 1990). Later work suggested that the MASH-1 gene may be a marker for early neuronal progenitors and that NGF causes the loss of this early marker in PC12 cells (Lo et al., 1991). Because of their possible significance for neuronal differentiation, it was of interest to study if MASH gene expression was regulated by TRα-1.

Northern blot analysis revealed no detectable expression of the MASH-2 gene (not shown). MASH-1 mRNA levels remained unchanged in normal and TRα-1 cells after 1 h treatment with NGF or NGF + T_3 (Fig. 6). Treatment for 24 or 144 h with these agents caused a significant decrease in MASH-1 mRNA levels in both cell types, although downregulation of MASH-1 message by NGF alone occurred more slowly in the TRα-1-expressing cells. These findings agree with those from Lo et al. (1991) and confirm our results on the early NGF-induced genes, suggesting that TRα-1 does not simply block NGF action, but causes a late arrest in PC12 differentiation via an NGF-independent pathway.

TRα-1 Prevents Entry of PC12 Cells into the Chromaffin Differentiation Pathway

Having shown that TRα-1 regulated neuronal differentiation and gene expression in a hormone-dependent fashion, we next investigated whether TRα-1 might affect the alternative differentiation pathway open to PC12 cells, i.e., chromaffin differentiation induced by dexamethasone (DEX). DEX was reported to cause morphological changes and expression of some chromaffin markers in PC12 cells (Greene and Tischler, 1982) and to antagonize the action of NGF at the molecular level (Leonard et al., 1987; Stein et al., 1988*b*).

After prolonged DEX treatment (20 d), uninfected PC12 cells adopted a rounded or polygonal shape, strongly adhering to each other, and formed cords and clumps which eventually fused into compacted epithelioid islands. This phenotype was not altered by the presence of T_3. In contrast, DEX-treated TRα-1 cells showed a delay in the formation of cell clumps which failed to develop into dense, compact islands. In presence of T_3, the cultures contained even more individual cells and few loose clumps, cells displaying a polygonal shape and short neurite-like spikes (data not shown).

These pilot studies raised the possibility that TRα-1 inhibited DEX-induced differentiation of PC12 cells into epithelioid, chromaffin-like cells. This was confirmed by the pattern of expression of epithelial markers such as cytokeratins and E-cadherin (uvomorulin). Strong expression of cytokeratins and weak, but distinct expression of membrane-bound uvomorulin was seen in the epithelioid islands obtained from DEX-treated uninfected cells. Expression of both markers was essentially missing in the loose groups of TRα-1-expressing cells subjected to the same treatment (data not shown, see below). We also studied the expression of the chromaffin marker TH. As expected, TH was strongly upregulated by DEX in uninfected cells (55–>98% strongly positive cells, Fig. 7 *A*). In contrast, TRα-1 cells expressed only low levels of TH (<10% weakly positive cells), which were only weakly upregulated by DEX (<30% positive cells; Fig. 7 *A*).

Differentiation of uninfected PC12 cells induced by prolonged DEX treatment was irreversible (Fig. 7 *B*). Even after DEX removal and subsequent treatment with NGF or NGF + T_3 most cells remained forming epithelioid islands expressing chromaffin markers (cytokeratin and uvomorulin). In contrast, TRα-1 expression prevented irreversible chromaffin differentiation. TRα-1 cells treated sequentially with DEX and NGF appeared immature with numerous short neurites and failed to express uvomorulin or cytokeratin (except for a low percentage of cytokeratin-positive cells; Fig. 7 *B*). As expected, if T_3 was administered together with NGF the cells differentiated into neurons. These results indicate that TRα-1-expressing PC12 cells respond abnormally to DEX induction. Instead of differentiating into chromaffin cells, they retain an undifferentiated phenotype and remain inducible by NGF + T_3 to form neurons. Thus TRα-1 constitutively prevents entry of PC12 cells into the chromaffin pathway, raising the possibility that TRα-1 expression commits the cells to the neuronal pathway of differentiation.

The effect of TRα-1 in blocking entry into the chromaffin pathway was also confirmed by studying the expression of two genes, TH and chromogranin B which although expressed in neurons are induced by DEX during chromaffin differentiation of PC12 cells (Lewis et al., 1983; Leonard et al., 1987; Stein et al., 1988*b*). Both basal and DEX-induced expression of TH was blocked in cells expressing TRα-1 (Figs. 6 and 8). Neither NGF nor T_3 or both could release the suppression of TH mRNA levels (Fig. 6). A much weaker suppressive effect of TRα-1 was observed for the chromogranin B and cytokeratin genes.

V-erbA Constitutively Induces an Aberrant Phenotype Combining Neuronal and Chromaffin Characteristics

The v-*erb*A oncogene acts as a constitutive repressor of red cell differentiation and erythrocyte gene expression in avian erythroblasts (Zenke et al., 1988, 1990; Disela et al., 1991). It also interferes with transactivation of synthetic TR/RAR

Published April 15, 1993

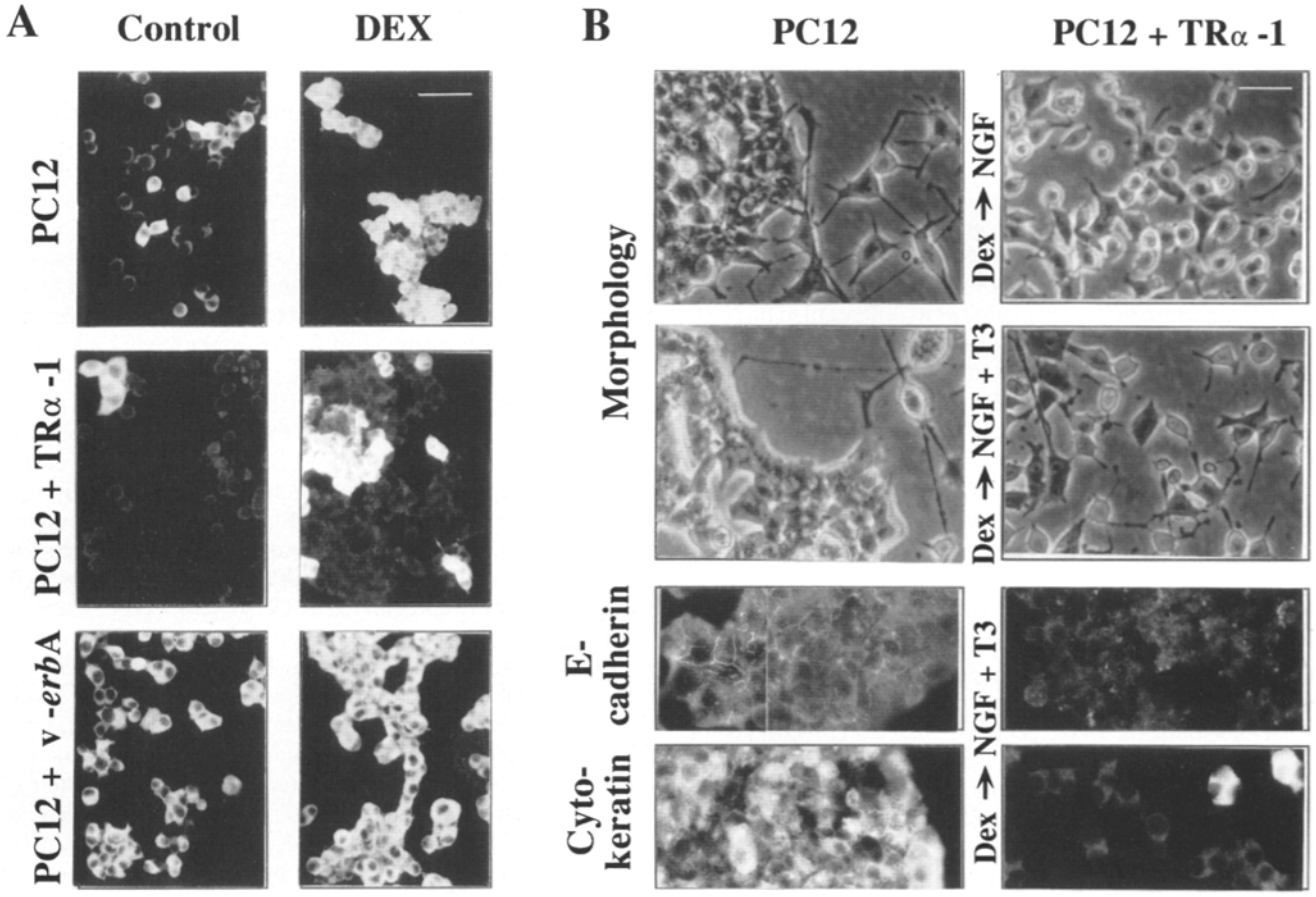

Figure 7. Effects of TRα–1 and v-*erb*A on dexamethasone-induced chromaffin differentiation in PC12 cells. (*A*) Immunofluorescence analysis of tyrosine hydroxylase protein expression in uninfected, TRα–1-, and v-*erb*A PC12 cells treated with dexamethasone for 2 wk (*DEX*) or left untreated (*Control.*) Bar, 50 μm. (*B*) (*Upper group of panels*; *Morphology*) Uninfected PC12 cells and TRα–1-PC12 cells were treated for 20 d with dexamethasone. They were then washed and incubated for an additional 7 d with NGF (Dex→NGF) or NGF + T_3 (DEX →NGF + T_3). Phase micrographs of representative areas are shown. Note presence of dense epithelioid islands surrounded by neuron-like cells in the normal PC12 cells. Bar, 50 μm. (*Lower group of panels*) Immunofluorescence analysis on parallel dishes to those photographed in the upper panels, using uvomorulin (*E-cadherin*) and cytokeratin antibodies, is shown.

response elements by RARα (Sharif and Privalsky, 1991) and has been claimed to prevent inhibition of fibroblast proliferation by RA (Desbois et al., 1991). We therefore studied in more detail how v-*erb*A affected differentiation and gene expression in PC12 cells.

As already described above, v-*erb*A expression caused a constitutive, partial inhibition of NGF-induced neuronal differentiation, regardless of the presence or absence of thyroid hormone (Fig. 3). In contrast to TRα-1 cells, DNA synthesis and cell proliferation were not affected by NGF or T_3 treatment in v-*erb*A cells (Fig. 4 *A*). Most strikingly, T_3 did not induce an NGF dependence for cell survival, and even the weak cytopathic effect induced by T_3 alone in uninfected cells was completely absent in v-*erb*A expressing cells (Fig. 4 *B*). This protective effect of v-*erb*A may well be due to suppression of endogenous c-*erb*A action.

Similarly to TRα-1, v-*erb*A did not affect the induction of early genes by NGF (Fig. 5). In contrast to TRα-1, however, v-*erb*A did not cause a repression of most late NGF inducible genes studied (Fig. 6) and was not able to prevent their induction by NGF. As expected from the inability of the v-*erb*A protein to bind and respond to hormone (Sap et al., 1986; Muñoz et al., 1988, 1990, see Fig. 1), T_3 did not alter expression levels of most NGF-inducible genes in v-*erb*A cells.

The transin (stromelysin) gene was an interesting exception. Induction of this gene by NGF was strongly inhibited in v-*erb*A cells as compared to normal PC12 cells (Fig. 6). This repression persisted even after incubation with NGF + T_3, leading to much lower levels of transin mRNA than obtained in normal or c-*erb*A cells treated similarly. It seems possible, therefore, that v-*erb*A is able to constitutively repress a subset of NGF-inducible genes, in line with its ability to partially inhibit neuronal differentiation.

Finally, v-*erb*A had an unexpected effect on DEX-induced chromaffin differentiation and gene expression. Treatment of v-*erb*A cells with DEX caused their flattening and aggregation into small groups, but epithelioid islands were not observed even after prolonged DEX treatment. In line with this finding, no E-cadherin could be detected on the cell membranes by immunofluorescence (data not shown). However, the cells expressed constitutively elevated levels of cytokeratin and TH. Even in untreated cells, staining with TH and cytokeratin antibodies was very strong (Fig. 7 *A* and data not shown) and even increased after DEX treatment (Fig. 7 *A*).

Published April 15, 1993

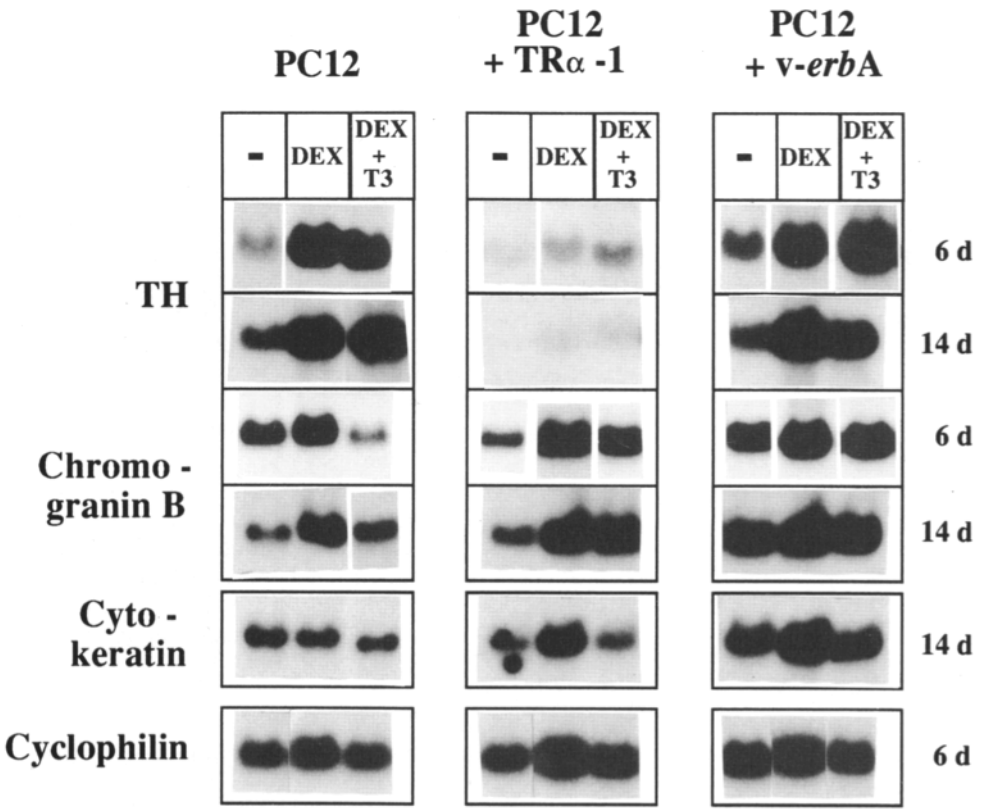

Figure 8. Induction of chromaffin and epithelial genes in *erb*A-expressing PC12 cells. Northern blot analyses of tyrosine hydroxylase (TH), chromogranin B, and cytokeratin mRNA expression was performed using uninfected, TRα-1, and v-*erb*A-PC12 cells. Cells were treated with DEX, DEX + T_3 for 6 (6 d) or 14 d (14 d), or left untreated (−). As controls, cyclophilin expression was determined. 10 μg of total RNA were loaded per lane. Exposure times: *TH*, 7 h; *Chromogranin* B, 4 h; 46 kd *Cytokeratin*, 3 d; and *Cyclophilin*, 2 d.

The results obtained for chromaffin marker gene expression were in line with these observations. Untreated or NGF-treated v-*erb*A cells expressed significantly higher levels of TH, chromogranin B, and cytokeratin messages than the respective uninfected cells (Fig. 8). However, DEX was still able to further induce these genes to similar levels in both normal and v-*erb*A-expressing cells. This suggests that v-*erb*A constitutively upregulates chromaffin marker gene expression, but does not prevent their regulation by DEX (Fig. 8). A summary of the effects of c-*erb*A/TRα-1 and v-*erb*A on PC12 cell differentiation and survival is represented in Fig. 9.

To rule out the trivial possibility that our v-*erb*A-PC12 cells fail to differentiate because of irreversible cell line progression changes, we tried to induce differentiation in

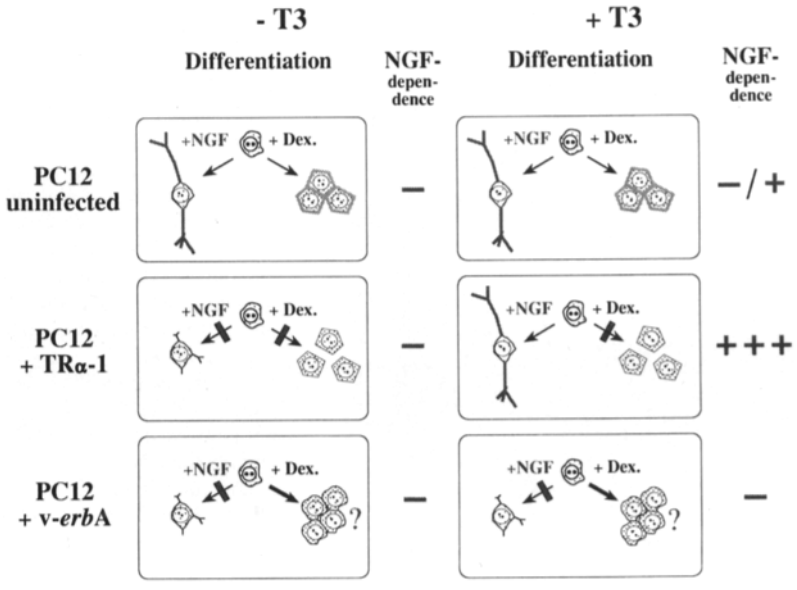

Figure 9. Schematic representation of how TRα-1 and v-*erb*A regulate PC12 cell differentiation into neurons and chromaffin cells. (*Upper panels*) In both presence and absence of T_3, normal PC12 cells differentiate into neurons or chromaffin cells upon NGF or dexamethasone treatment, respectively. They are independent of NGF for growth and survival in the absence of T_3 and exhibit a weak or absent NGF dependence (+/−) in its presence. (*Middle panels*) TRα-1-expressing PC12 cells are partially blocked (*black bar across arrow*) in their capacity to differentiate into either neurons or chromaffin cells in the absence of T_3. Upon T_3 addition, these cells respond normally to NGF, becoming neurons. In contrast, differentiation into more mature chromaffin cells remains blocked in the presence of T_3. TRα-1 cells are strongly dependent (+++) on NGF for survival when T_3 is present. (*Lower panels*) V-*erb*A-PC12 cells are partially blocked for NGF-induced neuronal differentiation regardless of the presence or absence of T_3. They exhibit a probably aberrant phenotype combining neuronal and chromaffin markers (indicated by altered cell shape) in the presence of dexamethasone. They do not respond at all to T_3 (−).

Published April 15, 1993

these cells by an alternative pathway. Cyclic AMP has been shown to induce part but not all of the NGF-induced phenotypic and gene expression changes in PC12 cells, most likely via pathway(s) independent of those important for NGF action (see Halegoua et al., 1991). Indeed, NGF plus dibutyryl-cAMP (0.1 mM) induced distinct morphological differentiation characterized by typical long neurites in both TRα-l- and v-*erb*A-expressing PC12 cells. In line with published work (Gunning et al., 1981), dibutyryl cyclic AMP alone at high concentrations (1 mM) also induced partial differentiation in both cell types. Thus, the v-*erb*A-induced differentiation arrest cannot be due to mutational changes in our cell lines.

Spontaneously Immortalized Rat Brain Neuroblasts Differentiate in Response to T_3 and NGF after Introduction of an Exogenous TRα-1 Gene

Since TRα-1 has no reported function in differentiation of normal adrenal medulla progenitors, the possibility remained that our results employing the PC12 cell system represented the aberrant behavior of a tumor cell line, mimicking normal differentiation processes as a consequence of artificial experimental treatments. We therefore sought to demonstrate an effect of TRα-1 in neuroblasts derived from the CNS, where the involvement of thyroid hormone receptors in neuronal maturation is amply documented (see introduction). Consequently, we introduced the avian TRα-1 into the E 18 cell line, which has been obtained by spontaneous immortalization from cultures of 17-d rat embryonic brain (Seliger, B., unpublished observations). E 18 cells represent primitive neuroblasts that express NF 68 and the primitive neuronal marker nestin, but lack the astrocyte marker, glial fibrillary acidic protein. After partial differentiation induction with dibutyryl-cAMP, the cells express further neuronal markers such as NF 145, NF 220, and neuron-specific enolase. Similarly to PC12 cells, the E18 cells expressed only low levels of TRα-1 together with high levels of TRα-2 (data not shown).

To test the ability of TRα-1 expressing E 18 cells to differentiate in response to NGF, T_3 or both, we used an E 18 clone infected with the TRα-1 retrovirus as described for PC12 cells. This clone expressed TRα-1 at similar levels as the TRα-1 PC12 clone cA4 (data not shown). As shown in Fig. 10, TRα-1 E 18 cells failed to differentiate in response to NGF alone, a property shared with uninfected E 18 cells. Also, NGF seemed not to influence E 18 cell proliferation. Upon T_3 addition, cells became less adherent and partially disintegrated (Fig. 10, T3) mirroring the cell killing effect observed in PC12 cells. In presence of both NGF and T_3, however, essentially all cells underwent massive neuronal differentiation characterized by outgrowth of long neurites

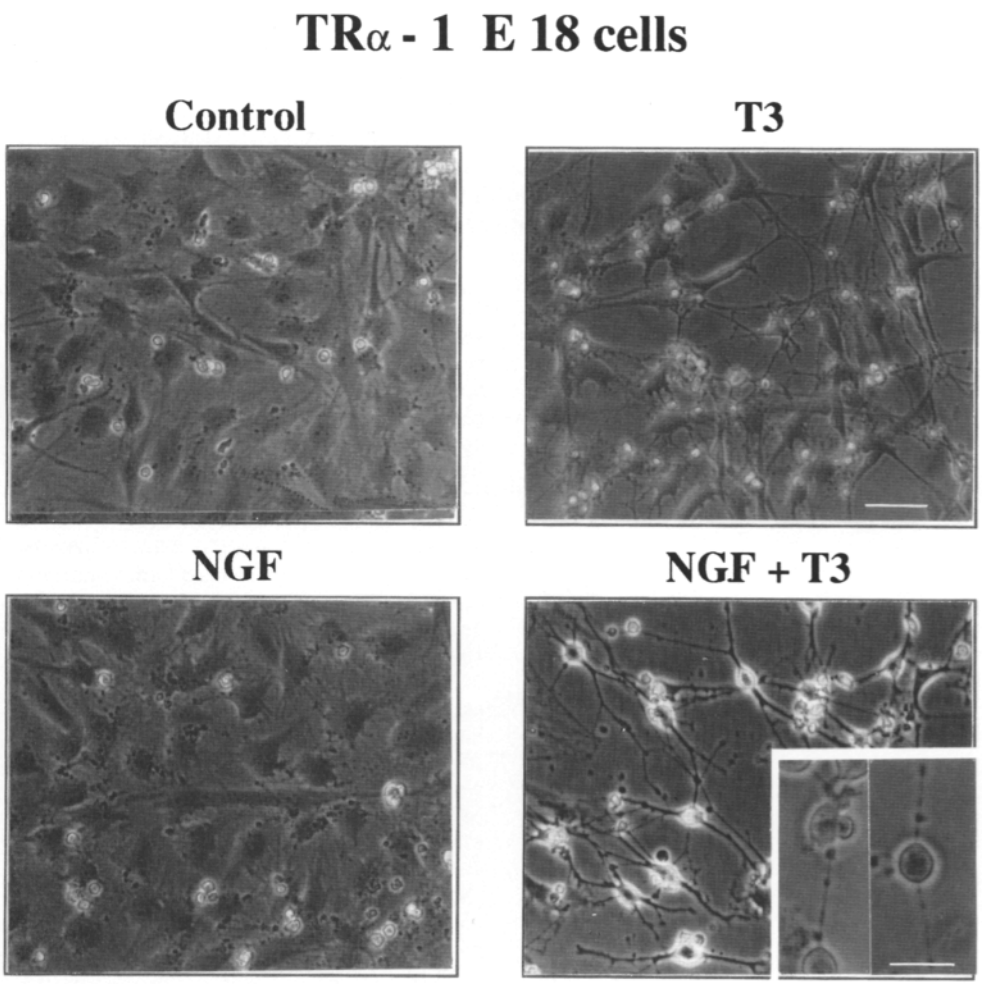

Figure 10. T_3 plus NGF induce differentiation in TRα-1 expressing E 18 neuroblasts derived from embryonic rat brain. Phase micrographs are shown from E 18 cA9 cells cultivated in serum-free differentiation medium for 6 d in absence (*Control*) or presence of T_3, NGF, or both (*NGF + T_3*). Note effective differentiation into neuron-like cells upon treatment with both NGF and T_3 (panel *NGF + T_3*) Inset: Some of the neuron-like cells obtained with NGF + T_3 in a separate experiment at higher magnification. Note also cell disintegration (refractile small bodies) in the T_3-treated cultures. Bar, 50 μm. Inset: bar, 20 μm.

Published April 15, 1993

within 6 d, often in a bipolar fashion (Fig. 10). Thus, TRα-1 expression in CNS derived neuroblasts conferred responsiveness to T_3 in a fashion very similar to that observed in TRα-1 expressing PC12 cells.

Discussion

Effect of TRα-1 on PC12 Cell Differentiation: Evidence for a NGF-independent, Permissive Function in Neuronal Differentiation

In this study, we provide the first direct evidence that TRα-1 controls neuronal differentiation and expression of neuron-specific genes in PC12 cells. Both NGF-induced neuronal differentiation and expression of late genes are arrested by TRα-1 in the absence of its ligand, T_3, while the block is released in the presence of the hormone, leading to efficient cell maturation (see Fig. 9 for a model). These effects of TRα-1 are strikingly similar to those observed in avian erythroblasts where TRα-1 regulates erythroid differentiation and erythrocyte gene expression in a T_3-dependent fashion (Zenke et al., 1990; Disela et al., 1991).

How does unliganded TRα-1 arrest neuronal differentiation? First, its action seems to be independent of NGF, since it did not affect expression of NGF-induced early genes. It also failed to inhibit NGF action completely since NGF-treated TRα-1-PC12 cells formed short neurites and lost expression of the early neuronal marker MASH-1. Rather, TRα-1 probably causes a late block in neuronal differentiation, perhaps by repressing or limiting the expression of indispensable, neuron-specific proteins. A possible candidate for such proteins is N-CAM, whose expression correlates very well with morphological differentiation of PC12 cells and causes neurite formation if expressed artificially in non-neuronal cells (Mann et al., 1989; Doherty et al., 1991). However, it is more likely that the observed block of differentiation is a combined result of the insufficient expression of many proteins with such diverse functions as maintenance of neurite structure or function and regulation of cell motility or cell-substrate adhesion.

The second important effect of TRα-1 was its ability to render differentiating PC12 cells dependent on NGF for survival. These results may correspond to the well-known fact that primary neurons differentiating in culture require neurotrophic factors for survival and neurite outgrowth. By such a mechanism, TRα-1 could control naturally occurring neuronal death during vertebrate development where large percentages (up to 80%) of certain neuronal populations undergo programmed cell death (Oppenheim, 1991). In line with this idea, thyroid hormone controls tissue regression and neuronal death during amphibian metamorphosis (Kollros, 1981; Tata, 1984; Tata et al., 1991) and the survival of cerebellar granule cells in hypothyroid rats (Lewis et al., 1976).

The effects of TRα-1 and thyroid hormone were not limited to PC12 cells. Studies on the E 18 cell line suggest that similar mechanisms are likely to be operating in vivo in selected neuronal populations of the CNS. In line with these findings we propose that TRα-1 controls neuronal differentiation in a permissive fashion: Neuronal progenitor cells are arrested at a certain stage of maturation by nonliganded TRα-1, until they are released to differentiate when ligand becomes available. At this stage, neurotrophic factors like NGF are required for survival of the differentiating cells. Thus, locally produced neurotrophic factors (NGF) and the systemic endocrine agent T_3 seem to cooperate in optimal induction of neuronal differentiation and neuronal gene expression. Cooperation of thyroid hormone and NGF has been demonstrated in vivo during development of the hippocampus, olfactory bulb, and cerebellum (Clos and Legrand, 1990), regions which express abundantly the TRα-1 isoform of T_3 receptor (Mellström et al., 1991).

In contrast to the erythroblast system (Schroeder et al., 1992*a*,*b*), TRα-1 does not seem to cooperate with RARs in neuronal differentiation of PC12 cells since the respective ligand (RA) did not affect this process or its regulation by TRα-1. Whether such a cooperation would occur upon artificial expression of RARs via retrovirus vectors or in mutant PC12 cells expressing elevated RAR levels (Scheibe et al., 1991) remains to be determined. The RARs might, however, be involved in regulating chromaffin differentiation, since RA induced morphological changes in PC12 cells resembling those induced by DEX. If this was true, the RARs might antagonize the function of TRα-1 rather than cooperating with it.

V-erbA Acts Like a Weak Constitutive Repressor of NGF-induced Differentiation and Gene Expression

In contrast to TRα-1, the v-*erb*A oncoprotein caused a partial, but constitutive block of NGF-induced PC12 cell differentiation. With the exception of the transin gene, expression of which was constitutively repressed by v-*erb*A, NGF regulation of other late genes studied was hardly affected by v-*erb*A. This could be due to the fact that v-*erb*A has a lower binding affinity to regulatory sequences of target genes (Sap et al., 1989; Disela et al., 1991). In addition, its expression level in PC12 cells was perhaps not high enough to efficiently repress the genes studied other than transin. Even in avian erythroblasts (the cell type in which v-*erb*A has been selected for its oncogenic activity) a large excess of v-*erb*A is necessary to repress genes activated by TRα-1/c-*erb*A or RARs (Disela et al., 1991; Schroeder et al., 1992*a*).

Our results suggest that v-*erb*A constitutively affects both lineages of PC12 cell differentiation. It partially arrests neuronal differentiation and gene expression, and also inhibits chromaffin differentiation although it is able to constitutively turn on some DEX-induced genes. As a result, v-*erb*A gives rise to an aberrant "interlineage" phenotype characterized by coexpression of certain neuronal and chromaffin markers (see Fig. 9 for a model). There are two nonexclusive possibilities to explain how v-*erb*A and TRα-1/c-*erb*A affect chromaffin differentiation and gene expression in such a different fashion. First, the point mutations in the v-*erb*A DNA binding domain might alter the DNA binding specificity of this mutated hormone receptor in that it now constitutively activates genes that are repressed by TRα-1. More likely, however, v-*erb*A differs from TRα-1/c-*erb*A in the way it interacts with other transcription factors such as RARs (Sharif and Privalsky, 1991, Desbois et al., 1991) or members of the AP-1 transcription factor family (Zhang et al., 1991) in transient transcription assays using standard cell lines. This functional difference between v-*erb*A and TRα-1 may both explain their different regulation of the chromaffin

Published April 15, 1993

marker gene tyrosine hydroxylase and their different effect on NGF-regulated proliferation of PC12 cells. Zhang et al. (1991) recently reported that T_3-activated c-*erb*A inhibits AP-1 activity in transiently transfected cells. This may explain why T_3 inhibits cell proliferation in TRα-1-PC12 cells. In addition, these authors showed that a truncated c-*erb*A carrying a COOH-terminal deletion very similar to that present in v-*erb*A was unable to block AP-1 activity, perhaps explaining why proliferation of our v-*erb*A-PC12 cells was not affected by T_3 (Fig. 4 *A*).

TRα-1-dependent Regulation of PC12 Differentiation: Relevance for Neuronal Differentiation in the CNS

Our results have several immediate consequences for the understanding of how thyroid hormone might act in the CNS. The observed permissive action of TRα-1 on neuron maturation could be an underlying mechanism for the abnormalities occurring in the hypothyroid rat brain. Unliganded TRα-1 might cause a late block in neurotrophin-dependent neuronal maturation. Similarly, the observation that the lesions can be reverted by T_3 administration during a critical period (Legrand, 1984) agrees well with the fact that T_3 releases the cells from this late block. By using the PC12 cell line approach, we were able to identify a good-sized number of candidate genes for regulation by T_3 and its receptor. Such genes may be impossible to detect by cDNA library screening approaches employing the whole brain or parts of it (Muñoz et al., 1991), since they may only occur in selected neuronal cell populations or during specific phases of brain development.

Although this work strongly suggests a direct, important role of thyroid hormone receptors in brain development, we are ignorant about mechanisms governing TR expression, the ratios between the different TR types, and the (likely) functional differences between the different TRs. Thus, studies like those presented here will have to be extended to other, preferably brain-derived neural cell types, to other TR types, and to the other neurotrophic factors recently identified (Barde et al., 1982; Leibrock et al., 1989; Lin et al., 1989; Hohn et al., 1990; Jones and Reichardt, 1990; Maisonpierre et al., 1990; Berkemeier et al., 1991; Hallböök et al., 1991). An obvious choice for such systems will be neuroblast lines such as the E 18 line described here. Due to the instability of such lines and their reported ability to change their phenotype (e.g., from neuronal to glial, Gage, 1992), more work is required before such lines can be used for molecular studies in the fashion described here for the PC12 system.

We want to thank those mentioned in Materials and Methods for generously supplying us with plasmids or antibodies, and Dr. E. Reichmann and E. Deiner for their help with the immunofluorescence experiments. We are also grateful to Dr. M. Busslinger for fruitful discussions and for critically reading the manuscript.

Received for publication 11 September 1992 and in revised form 22 December 1992.

References

Anderson, D. J., and R. Axel. 1986. A bipotential neuroendocrine precursor whose choice of cell fate is determined by NGF and glucocorticoids. *Cell.* 47:1079–1090.

Barde, Y. A., D. Edgar, and H. Thoenen. 1982. Purification of a new neurotrophic factor from mammalian brain. *EMBO (Eur. Mol. Biol. Organ.) J.* 1:549–553.

Bartel, D. P., M. Sheng, L. F. Lau, and M. E. Greenberg. 1989. Growth factors and membrane depolarization activate distinct programs of early response gene expression: dissociation of *fos* and *jun* induction. *Genes & Dev.* 3:304–313.

Bernal, J., and F. Pekonen. 1984. Ontogenesis of the nuclear 3,5,3′-triiodothyronine receptor in the human fetal brain. *Endocrinology.* 114: 677–679.

Berkemeier, L. R., J. W. Winslow, D. R. Kaplan, K. Nikolics, D. V. Goeddel, and A. Rosenthal. 1991. Neurotrophin-5: a novel neurotrophic factor that activates *trk* and *trk*B. *Neuron.* 7:857–866.

Beug, H., P. A. Blundell, and T. Graf. 1987. Reversibility of differentiation and proliferation capacity in avian myelomonocytic cells transformed by *ts* E26 leukemia virus. *Genes & Dev.* 1:277–286.

Beug, H., G. Doederlein, and M. Zenke. 1992. Transformation by v-*erb*A and v-*erb*B oncogenes: independent modulation of differentiation and proliferation in erythroid progenitors. *In* Bristol Myers Symposia on Nuclear Processes and Oncogenes. Vol. 15. P. A. Sharp, editor. Academic Press, Orlando, FL. 53–84.

Bottenstein, J. E., and G. H. Sato. 1979. Growth of a rat neuroblastoma cell line in serum-free supplemented medium. *Proc. Natl. Acad. Sci. USA.* 76:514–517.

Bradley, D. J., W. S. Young III, and C. Weinberger. 1989. Differential expression of α and β thyroid hormone receptor genes in rat brain and pituitary. *Proc. Natl. Acad. Sci. USA.* 86:7250–7254.

Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156–159.

Church, G. M., and W. Gilbert. 1984. Genome sequencing. *Proc. Natl. Acad. Sci. USA.* 81:1991–1995.

Clos, J., and Ch. Legrand. 1990. An interaction between thyroid hormone and nerve growth factor promotes the development of hippocampus, olfactory bulbs and cerebellum: a comparative biochemical study of normal and hypothyroid rats. *Growth Factors.* 3:205–220.

Costello, B., A. Meymandi, and M. C. Freeman. 1990. Factors influencing GAP-43 gene expression in PC12 pheochromocytoma cells. *J. Neurosci.* 10:1398–1406.

Damm, K., C. C. Thompson, and R. M. Evans. 1989. Protein encoded by v-*erb*A functions as a thyroid hormone receptor antagonist. *Nature (Lond.).* 339:593–597.

Desbois, C., B. Pain, C. Guilhot, M. Benchaibi, M. French, J. Ghysdael, J.-J. Madjar, and J. Samarut. 1991. v-*erb*A oncogene abrogates growth inhibition of chicken embryo fibroblasts induced by retinoic acid. *Oncogene.* 6:2129–2135.

Disela, C., C. Glineur, T. Bugge, J. Sap, G. Stengl, J. Dogson, H. Stunnenberg, H. Beug, and M. Zenke. 1991. V-*erb*A overexpression is required to extinguish c-*erb*A function in erythroid cell differentiation and regulation of the *erb*A target gene CAII. *Genes & Dev.* 5:2033–2047.

Doherty, P., L. H. Rowett, S. E. Moore, D. A. Mann, and F. S. Walsh. 1991. Neurite outgrowth in response to transfected N-CAM and N-cadherin reveals fundamental differences in neuronal responsiveness to CAMs. *Neuron.* 6:247–258.

Doupe, A. J., S. C. Landis, and P. H. Patterson. 1985*a*. Environmental influences in the development of neuronal crest derivatives: glucocorticoids, growth factors, and chromaffin cell plasticity. *J. Neurosci.* 5:2119–2142.

Doupe, A., P. H. Patterson, and S. C. Landis. 1985*b*. Small intensely fluorescent cells in culture: role of glucocorticoids and growth factors in their development and interconversions with neural crest derivatives. *J. Neurosci.* 5:2143–2160.

Dussault, J. H., and J. Ruel. 1987. Thyroid hormones and brain development. *Annu. Rev. Physiol.* 49:321–334.

Falcone, G., D. Boettiger, S. Alema, and F. Tato. 1984. Role of cell division in differentiation of myoblasts infected with a temperature-sensitive mutant of Rous sarcoma virus. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:1327–1331.

Federoff, H. J., E. Grabczyk, and M. C. Fishman. 1988. Dual regulation of GAP-43 gene expression by nerve growth factor and glucocorticoids. *J. Biol. Chem.* 263:19290–19295.

Feinberg, P. A., and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 137:266–267.

Forrest, D., M. Sjorberg, and B. Vennström. 1990. Contrasting developmental and tissue-specific expression of α and β thyroid hormone receptor genes. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:1519–1528.

Forrest, D., F. Hallböök, H. Persson, and B. Vennström. 1991. Distinct functions for thyroid hormone receptors α and β in brain development indicated by differential expression of receptor genes. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:269–275.

Gage, F. H. 1992. Repopulating the mortal brain with immortal cells. *Current Biology.* 2:232–234.

Glineur, C., M. Zenke, H. Beug, and J. Ghysdael. 1990. Phosphorylation of v-*erb*A is required for its function as an oncogene. *Genes & Dev.* 4:1663–1676.

Goldberg, Y., C. Glineur, J.-C. Gesquiere, A. Ricouart, J. Sap, B. Vennström, and J. Ghysdael. 1988. Activation of protein kinase C or cAMP-dependent

protein kinase increases phosphorylation of the c-*erb*A-encoded thyroid hormone receptor and of the v-*erb*A-encoded protein. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:2425–2433.

Gould, E., and L. L. Butcher. 1989. Developing cholinergic basal forebrain neurons are sensitive to thyroid hormone. *J. Neurosci.* 9:3347–3358.

Greenberg, M. E., L. A. Greene, and E. B. Ziff. 1985. Nerve growth factor and epidermal growth factor induce rapid transient changes in proto-oncogene transcription in PC12 cells. *J. Biol. Chem.* 260:14101–14110.

Greene, L. A., and A. S. Tischler. 1976. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma which respond to nerve growth factor. *Proc. Natl. Acad. Sci. USA.* 73:2424–2428.

Greene, L. A., and A. S. Tischler. 1982. PC12 pheochromocytoma cultures in neurobiological research. *Adv. Cell. Neurobiol.* 3:373–414.

Gunning, P. W., G. E. Landreth, M. A. Bothwell, and E. M. Shooter. 1981. Differential and synergistic actions of nerve growth factor and cyclic AMP in PC12 cells. *J. Cell Biol.* 89:240–245.

Halegoua, S., R. C. Armstrong, and N. E. Kremer. 1991. Dissecting the mode of action of a neuronal growth factor. *In* Current Topics in Microbiology and Immunology. Neuronal Growth Factors. M. Bothwell, editor. Springer-Verlag, Berlin/Heidelberg. 119–170.

Hallböök, F., C. F. Ibañez, and H. Persson. 1991. Evolutionary studies of the nerve growth factor family reveal a novel member abundantly expressed in Xenopus ovary. *Neuron.* 6:845–858.

Hamburg, M. 1969. The role of thyroid hormone and growth hormones in neurogenesis. *In* Current Topics in Developmental Biology 4, A. Moscona and A. Monroy, editors. Academic Press, New York. 109–148.

Hohn, A., J. Leibrock, K. Bailey, and Y.-A. Barde. 1990. Identification and characterization of a novel member of the nerve growth factor/brain-derived neurotrophic factor family. *Nature (Lond.).* 344:339–341.

Holtzman, D. M., Y. Li, L. F. Parada, S. Kinsman, C.-K. Chen, J. S. Valletta, J. Zhou, J. B. Long, and W. C. Mobley. 1992. p140^{trk} mRNA marks NGF-responsive forebrain neurons: evidence that *trk* gene expression is induced by NGF. *Neuron.* 9:465–478.

Hudson, L. G., J. B. Santon, C. K. Glass, and J. A. Gill. 1990. Ligand-activated thyroid hormone and retinoic acid receptors inhibit growth factor receptor promoter expression. *Cell.* 62:1165–1175.

Ito, E., L. A. Sweterlitsch, P. B.-V. Tran, F. J. Rauscher III, and R. Narayanan. 1990. Inhibition of PC12 cell differentiation by the immediate early gene *fra*-1. *Oncogene.* 5:1755–1760.

Johnson, J. E., S. J. Birren, and D. J. Anderson. 1990. Two rat homologues of Drosophila *achaete-scute* specifically expressed in neuronal precursors. *Nature (Lond.).* 346:858–861.

Jones, K. R., and L. J. Reichardt. 1990. Molecular cloning of a human gene that is a member of the nerve growth factor family. *Proc. Natl. Acad. Sci. USA.* 87:8060–8064.

Koenig, R. J., M. A. Lazar, R. A. Hodin, G. A. Brent, P. R. Larsen, W. W. Chin, and D. D. Moore. 1989. Inhibition of thyroid hormone action by a non-hormone binding c-*erb*A protein generated by alternative mRNA splicing. *Nature (Lond.).* 337:659–661.

Kollros, J. J. 1981. Transitions in the nervous system during amphibian metamorphosis. *In* Metamorphosis: A Problem in Developmental Biology. L. I. Gilbert and E. Frieden, editors. Plenum Press, New York. 445–459.

Kruijer, W., D. Schubert, and M. I. Verma. 1985. Induction of proto-oncogene *fos* by nerve growth factor. *Proc. Natl. Acad. Sci. USA.* 82:7330–7334.

Lazar, M. A., R. A. Hodin, and W. W. Chin. 1989. Human carboxyl-terminal variant of a α-type c-*erb*A inhibits trans-activation by thyroid hormone receptors without binding thyroid hormone. *Proc. Natl. Acad. Sci. USA.* 86:7771–7774.

Legrand, J. 1984. Effects of thyroid hormones on central nervous system. *In* Neurobehavioural Teratology. J. Yanai, editor. Elsevier, Amsterdam. 331–363.

Leibrock, J., F. Lottspeich, A. Hohn, M. Hofer, B. Hengerer, P. Masiakowski, H. Thoenen, and Y. A. Barde. 1989. Molecular cloning and expression of brain-derived neurotrophic factor. *Nature (Lond.).* 341:149–152.

Leonard, D. G. B., L. B. Ziff, and L. A. Greene. 1987. Identification and characterization of mRNAs regulated by nerve growth factor in PC12 cells. *Mol. Cell Biol.* 7:3156–3167.

Levi, A., S. Biocca, A. Cattaneo, and P. Calissano. 1988. The mode of action of nerve growth factor in PC12 cells. *Mol. Neurobiol.* 2:201–226.

Lewis, P. D., A. J. Patel, A. L. Johnson, and R. Balazs. 1976. Effect of thyroid deficiency on cell acquistition in the postnatal rat brain: a quantitative histological study. *Brain Res.* 104:49–62.

Lewis, E. J., A. W. Tank, N. Weiner, and D. Chikaraishi. 1983. Regulation of tyrosine hydroxylase mRNA by glucocorticoid and cyclic AMP in a rat pheochromocytoma cell line. *J. Biol. Chem.* 258:14,632–14,637.

Lin, L. F., D. Mismer, J. D. Lile, L. G. Armes, E. T. Butler III, J. L. Vannice, and F. Collins. 1989. Purification, cloning and expression of ciliary neurotrophic factor. *Science (Wash. DC).* 246:1023–1025.

Lindenbaum, M. H., S. Carbonetto, F. Grosveld, D. Flavell, and W. E. Mushynski. 1988. Transcriptional and post-transcriptional effects of nerve growth factor on expression of the three neurofilament subunits in PC12 cells. *J. Biol. Chem.* 263:5662–5667.

Lo, L.-C., J. E. Johnson, C. Wuenschell, T. Saito, and D. J. Anderson. 1991. Mammalian *achaete-scute* homolog 1 is transiently expressed by spatially restricted subsets of early neuroepithelial and neural crest cells. *Genes & Dev.* 5:1524–1537.

Machida, C. M., K. D. Rodland, L. Matrisian, B. E. Magun, and G. Ciment. 1989. NGF induction of the gene encoding the protease transin accompanies neuronal differentiation in PC12 cells. *Neuron.* 2:1587–1596.

Maisonpierre, P. C., L. Belluscio, S. Squinto, N. Y. Ip, M. E. Furth, R. M. Lindsay, and G. D. Yancopoulos. 1990. Neurotrophin-3: a neurotrophic factor related to NGF and BDNF. *Science (Wash. DC).* 247:1446–1451.

Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 545 pp.

Mann, D. A., P. Doherty, and F. S. Walsh. 1989. Increased intracellular cyclic AMP differentially modulates nerve growth factor induction of three neuronal recognition molecules involved in neurite outgrowth. *J. Neurochem.* 53:1581–1588.

Mellström, B., J. R. Naranjo, A. Santos, A. M. González, and J. Bernal. 1991. Independent expression of the α and β c-*erb*A genes in developing rat brain. *Mol. Endocrinol.* 5:1339–1350.

Milbrandt, J. 1987. A nerve growth factor-induced gene encodes a possible transcriptional regulatory factor. *Science (Wash. DC).* 238:797–799.

Milbrandt, J. 1988. Nerve growth factor induces a gene homologous to the glucocorticoid receptor gene. *Neuron.* 1:183–188.

Morgan, J. I., and T. Curran. 1986. Role of ion flux in the control of c-*fos* expression. *Nature (Lond.).* 322:552–555.

Morreale de Escobar, G., F. Escobar del Rey, and A. Ruiz-Marcos. 1983. Thyroid hormone and the developing brain. *In* Congenital Hypothyroidism. J. H. Dussault and P. Walker, editors. Marcel Decker, New York. 85–126.

Muñoz, A., M. Zenke, U. Gehring, J. Sap, H. Beug, and B. Vennström. 1988. Characterization of the hormone-binding domain of the chicken c-*erb*A/thyroid hormone receptor protein. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:155–159.

Muñoz, A., W. Höppner, J. Sap, G. Brady, K. Nordström, H. J. Seitz, and B. Vennström. 1990. The chicken c-*erb*A-α-product induces expression of thyroid hormone-responsive genes in 3,5,3′-triiodothyronine receptor-deficient rat hepatoma cells. *Mol. Endocrinol.* 4:312–320.

Muñoz, A., A. Rodríguez-Peña, A. Pérez-Castillo, B. Ferreiro, J. G. Sutcliffe, and J. Bernal. 1991. Effects of neonatal hypothyroidism on rat brain gene expression. *Mol. Endocrinol.* 5:273–280.

Oppenheim, R. W. 1991. Cell death during development of the nervous system. *Annu. Rev. Neurosci.* 14:453–501.

Patel, A. J., M. Hayashi, and A. Hunt. 1988. Role of thyroid hormone and nerve growth factor in the development of choline acetyltransferase and other cell-specific marker enzymes in the basal forebrain of the rat. *J. Neurochem.* 50:803–811.

Prentice, H. M., S. E. Moore, J. G. Dickson, P. Doherty, and F. S. Walsh. 1987. Nerve growth factor-induced changes in neural cell adhesion molecule (N-CAM) in PC12 cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:1859–1863.

Rentoumis, A., V. Krishna, K. Chatterjee, L. D. Madison, S. Datta, G. D. Gallagher, L. J. DeGroot, and J. L. Jameson. 1990. Negative and positive transcriptional regulation by thyroid hormone receptor isoforms. *Mol. Endocrinol.* 4:1522–1531.

Samuels, J. J., F. Stanley, and L. E. Shapiro. 1976. Dose-dependent depletion of nuclear receptor levels by L-triiodothyronine: evidence for a role in induction of growth hormone synthesis in cultured GH1 cells. *Proc. Natl. Acad. Sci. USA.* 73:3877–3881.

Samuels, J. J., F. Stanley, and L. E. Shapiro. 1977. Modulation of thyroid hormone nuclear receptor levels by 3,5,3′-triiodothyronine in GH1 cells. Evidence for two functional components of nuclear-bound receptor and relationship to the induction of growth hormone synthesis. *J. Biol. Chem.* 252: 6052–6060.

Sap, J., A. Muñoz, K. Damm, Y. Goldberg, J. Ghysdael, A. Leutz, H. Beug, and B. Vennström. 1986. The c-*erb*A protein is a high affinity receptor for thyroid hormone. *Nature (Lond.).* 324:635–640.

Sap, J., A. Muñoz, J. Schmitt, H. Stunnenberg, and B. Vennström. 1989. Repression of transcription mediated at a thyroid hormone response element by the v-*erb*A oncogene product. *Nature (Lond.).* 340:242–244.

Scheibe, R. J., D. D. Ginty, and J. O. Wagner. 1991. Retinoic acid stimulates the differentiation of PC12 cells that are deficient in cAMP-dependent protein kinase. *J. Cell Biol.* 113:1173–1182.

Schroeder, C., L. Gibson, and H. Beug. 1992*a*. The v-*erb*A oncogene requires cooperation with tyrosine kinases to arrest erythroid differentiation induced by ligand activated endogenous c-*erb*A and retinoic acid receptor. *Oncogene.* 7:203–216.

Schroeder, C., L. Gibson, M. Zenke, and H. Beug. 1992*b*. Modulation of normal erythroid differentiation by the endogenous thyroid hormone and retinoic acid receptors: a possible target for v-*erb*A oncogene action. *Oncogene.* 7:217–227.

Sharif, M., and M. Privalsky. 1991. V-erbA oncogene function in neoplasia correlates with its ability to repress retinoic acid receptor action. *Cell.* 66:1–20.

Stein, R., N. Mori, K. Matthews, L. C. Lo, and D. J. Anderson. 1988*a*. The NGF-inducible SCG10 mRNA encodes a novel membrane-bound protein present in growth cones and abundant in developing neurons. *Neuron.* 1:463–476.

Stein, R., S. Orit, and D. J. Anderson. 1988*b*. The induction of a neural-specific gene, SCG10, by nerve growth factor in PC12 cells is transcriptional, pro-

tein synthesis dependent, and glucocorticoid inhibitable. *Dev. Biol.* 127: 316–325.

Strait, K. A., H. L. Schwartz, V. S. Seybold, N. C. Ling, and J. H. Oppenheimer. 1991. Immunofluorescence localization of thyroid hormone receptor protein β1 and variant α2 in selected tissues: cerebellar Purkinje cells as a model for β1 receptor-mediated developmental effects of thyroid hormone in brain. *Proc. Natl. Acad. Sci. USA.* 88:3887–3891.

Szeberényi, J., H. Cai, and G. M. Cooper. 1990. Effect of a dominant inhibitory Ha-*ras* mutation on neuronal differentiation of PC12 cells. *Mol. Cell Biol.* 10:5324–5332.

Tata, J. R. 1984. The action of growth and developmental hormones: evolutionary aspects. *In* Biological Regulation and Development. Vol. 3B, R. F. Goldberger and K. R. Yamamoto, editors. Plenum Press, New York. 1–58.

Tata, J.R., A. Kawahara, and B. S. Baker. 1991. Prolactin inhibits both thyroid hormone-induced morphogenesis and cell death in cultured amphibian larval tissues. *Dev. Biol.* 146:72–80.

Van Hoof, C. O., J. C. Holthuis, A. B. Oestriecher, J. Boonstra, P. N. De Graan, and W. H. Gispen. 1989. Nerve growth factor-induced changes in the intracellular localization of the protein kinase C substrate B-50 in pheochromocytoma PC12 cells. *J. Cell Biol.* 108:1115–1125.

Vennström, B., and J. M. Bishop. 1982. Isolation and characterization of chicken DNA homologous to the two putative oncogenes of avian erythroblastosis virus. *Cell.* 28:135–143.

Weinberger, C., C. C. Thompson, E. S. Ong, R. Lebo, D. J. Gruol, and R. M. Evans. 1986. The c-*erb*A encodes a thyroid hormone receptor. *Nature (Lond.).* 324:641–646.

Zenke, M., P. Kahn, C. Disela, B. Vennström, A. Leutz, K. Keegan, M. J. Hayman, H.-R. Choi, N. Yew, J. D. Engel, and H. Beug. 1988. v-*erb*A specifically suppresses transcription of the avian erythrocyte anion transporter (band 3) gene. *Cell.* 52:107–119.

Zenke, M., A. Muñoz, J. Sap, B. Vennström, and H. Beug. 1990. v-*erb*A oncogene activation entails the loss of hormone-dependent regulator activity of c-*erb*A. *Cell.* 61:1035–1049.

Zhang, X.-K., K. N. Wills, M. Husmann, T. Hermann, and M. Pfahl. 1991. Novel pathway for thyroid hormone receptor action through interaction with *jun* and *fos* oncogene activities. *Mol. Cell. Biol.* 11:6016–6025.